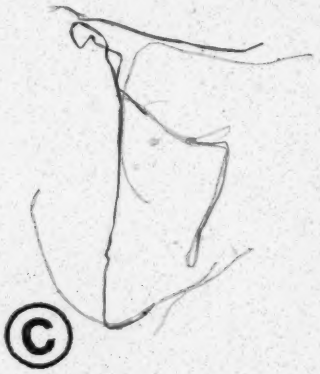
University of Alberta

Investigations of Bacteriocins from *Lactococcus lactis* and *Bacillus subtilis*

by

Lara Silkin



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ABSTRACT

Bacteriocins are ribosomally synthesized antimicrobial peptides that are typically active against bacteria closely related to the producer strain. Lacticin 3147 is a two-component bacteriocin consisting of the peptides LtnA1 and LtnA2, both of which contain D-alanine residues. In lacticin 3147, these residues are formed by LtnJ in the enzymatic reduction of 2,3-dehydroalanine.

LtnJ was subcloned and overexpressed as hexahistidine and maltose binding protein (MBP) fusions in *E. coli*. Three 2,3-dehydroalanine containing dipeptides and three 2,3-dehydroalanine containing tripeptides were synthesized, each containing an N-acyl group and a C-terminal methyl ester, to model the sequences of LtnA1 and LtnA2. The development of an activity assay for LtnJ was attempted using partially purified enzyme from hexahistidine and MBP fusions, the synthesized dipeptides and tripeptides as substrates, and various co-factor combinations.

The three bacteriocins lacticin 3147, nisin A, and subtilosin A were tested as spermicides against bovine, horse/pony, pig, and rat sperm.

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Dedicated to my mother and father

TABLE OF CONTENTS

	Page
CHAPTER 1. INTRODUCTION TO BACTERIOCINS	
1.1. The Bacterial Cell Wall and Antibiotics	1
1.2. Bacteriocins	4
1.2.1. Subtilosin A	7
1.3. Lantibiotics	9
1.3.1. Nisin	14
1.3.2. Lacticin 3147	20
1.4. Objectives	27
CHAPTER 2. LTNJ, A MODIFICATION ENZYME IN LACTICIN 3147	
2.1. The Bacteriocin Producing Plasmid pMRC01 from *L. lactis* subsp. *lactis* DPC3147	29
2.2. Sequence Homology of LtnJ	30
2.3. Introduction of LtnJ in *E. coli* Expression Systems	32
2.4. Isolation of LtnJ from *E. coli* Containing pPC1 or pPC2	35
2.5. Synthesis of Dipeptides	41
2.6. Testing Dipeptides as Substrates of LtnJ from *E. coli* BL21(DE3) Containing pPC1	43
2.7. Formation and Screening of New *E. coli* LtnJ Overexpression Strains	47
2.8. Optimization of Expression Strains for pLS3	54
2.9. Purification of LtnJ from *E. coli* K12 ER2507 Containing pLS3	57
2.10. Testing Dipeptides as Substrates of LtnJ from *E. coli* K12 ER2507 Containing pLS3	60

2.11. LtnJ from the Cell Free Extract of *L. lactis* 63

2.12. Testing Dipeptides as Substrates of LtnJ from CFE of *L. lactis* 64

2.13. Synthesis of Tripeptides 64

2.14. Testing Tripeptides as Substrates of LtnJ from CFE of *L. lactis* 70

2.15. Testing Tripeptides as Substrates of LtnJ from *E. coli* K12 ER2507 Containing pLS3 84

2.16. Isolation of Lacticin 3147 from an *L. lactis* Δ*ltnJ* Mutant 84

2.17. Testing Lacticin 3147 Mutants as Substrates of LtnJ from CFE of *L. lactis* 85

2.18. Conclusions and Future Directions 86

CHAPTER 3. ANTIMICROBIAL AGENTS AS SPERMICIDES

3.1. Microbicides and Spermicides 88

3.2. The Spermicidal Properties of Nisin 90

3.3. The Testing of Bacteriocins as Spermicides 92

3.4. Conclusions and Future Directions 100

CHAPTER 4. EXPERIMENTAL PROCEDURES

4.1. Synthesis of Dipeptides and Tripeptides 104

GENERAL METHODOLOGIES

4.1.1. Reagents, Solvents, and Solutions 104

4.1.2. Purification Techniques 104

4.1.3. Instrumentation for Compound Characterization 105

GENERAL PROCEDURES FOR COMPOUND PREPARATIONS

4.1.4. General Procedure for Preparation of Dehydroalanine Containing Dipeptides (**24-26**) 106

4.1.5. General Procedure for Preparation of Protected D-Alanine Containing Dipeptides (**31-33**) 106

4.1.6. General Procedure for Preparation of D-Alanine Containing Dipeptides (**34-36, 60**) 107

4.1.7. General Procedure for Preparation of Protected Serine Containing Dipeptides (**38, 45, 46**) 108

4.1.8. General Procedure for Preparation of Protected Tripeptides (**49, 50, 55-57**) 108

4.1.9. General Procedure for Preparation of Protected Dehydro Alanine Containing Tripeptides (**42, 51, 52**) 109

4.1.10. General Procedure for Preparation of Acyl Tripeptides (**43, 53, 54, 58, 59**) 109

4.1.11. Experimental Data for Compounds 110

(*S*)-Methyl 2-(2-acetamidoacrylamido)propanoate (**24**) 110

(*S*)-Methyl 2-(2-acetamidoacrylamido)-4-methylpentanoate (**25**) 111

(2*S*,3*R*)-Methyl 2-(2-acetamidoacrylamido)3-methylpentanoate (**26**) 112

(*S*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)propanoate (**31**) 112

(*S*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)-4-methylpentanoate (**32**) 113

(2*S*,3*R*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-methylpentanoate (**33**) 114

(*S*)-Methyl 2-((*R*)-2-acetamidopropanamido)propanoate (**34**) 114

(*S*)-Methyl 2-((*R*)-2-acetamidopropanamido)-4-methylpentanoate (**35**) 115

(2*S*,3*R*)-Methyl 2-((*R*)-2-acetamidopropanamido)-3-methylpentanoate (**36**) 116

(*S*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido) propanoate (**38**) 116

(6*S*,9*S*,12*S*)-Methyl 9-(hydroxymethyl)-6-isobutyl-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecane-12-carboxylate (**41**) 117

(*S*)-Methyl 6-isobutyl-2,2-dimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazatridecane-12-carboxylate (**42**) 119

(*S*)-Methyl 2-(2-((*S*)-2-acetamido-4-methylpentanamido)acrylamido) propanoate (**43**) 120

(*S*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)-4-methylpentanoate (**45**) 121

(2*S*,3*R*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)-3-methylpentanoate (**46**) 121

(6*S*,9*S*,12*S*)-Methyl 6-benzyl-9-(hydroxymethyl)-2,2,14-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (**49**) 122

(6*S*,9*S*,12*S*,13*R*)-Methyl 6-*sec*-butyl-9-(hydroxymethyl)-2,2,13-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (**50**) 123

(*S*)-Methyl 6-benzyl-2,2,14-trimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (**51**) 124

(6*S*,12*S*,13*R*)-Methyl 6-*sec*-butyl-2,2,13-trimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (**52**) 125

(*S*)-Methyl 2-(2-(2-acetamido-3-phenylpropanamido)acrylamido)-4-methylpentanoate (**53**) 126

(2*S*,3*R*)-Methyl 2-(2-((2S,3S)-2-acetamido-3-methylpentanamido)acrylamido)-3-methylpentanoate (**54**) 127

(6*S*,9*R*,12*S*)-Methyl 6-isobutyl-2,2,9,12-tetramethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (**55**) 128

(6*S*,9*R*,12*S*)-Methyl 6-benzyl-12-isobutyl-2,2,9-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (**56**) 129

(6*S*,9*R*,12*S*)-Methyl 6,12-di-*sec*-butyl-2,2,9-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (**57**) 130

(*S*)-Methyl 2-((*R*)-2-((*S*)-2-acetamido-4-methylpentanamido)propanamido) propanoate (**58**) 131

(*S*)-Methyl 2-((*R*)-2-((*S*)-2-acetamido-3-phenylpropanamido)propanamido)-4-methylpentanoate (**59**) 132

(2*S*,3*R*)-Methyl 2-((*R*)-2-((2*S*,3*S*)-2-acetamido-3-methylpentanamido)propanamido)-3-methylpentanoate (**60**) 133

4.2. Lacticin 3147 LtnJ 134

4.2.1. Bacterial Strains, Plasmids, and Culture Media 134

4.2.2. DNA Manipulations and Transformations 136

4.2.3. Purification of Recombinant Proteins 137

4.2.4. SDS-PAGE, Western Blot, and Dot Blot Analysis 137

4.2.5. Transformation of *E. coli* 139

4.2.6. Plasmid Isolation and Sequencing 139

4.2.7. Construction of Plasmid pLS1A 140

4.2.8. Construction of Plasmid pLS1B 142

4.2.9. Construction of Plasmid pLS2 144

4.2.10. Construction of Plasmid pLS3 144

4.2.11. Detection of Soluble Production of LtnJ from *E. coli* BL21(DE3) and Origami™B(DE3) Containing pPC1 or pPC2 at Different Temperatures 146

4.2.12. Detection of Soluble Production of LtnJ from *E. coli* BL21(DE3) Containing pLS1A, pLS1B, or pLS2 and *E. coli* K12 TB1, K12 JM101, K12 CAG629, K12 UT5600, K12 ER2507, and K12 ER2508 Containing pLS3 at Different Temperatures 147

4.2.13. Isolation of the N-terminal Histidine Tagged LtnJ from *E. coli* BL21(DE3) Containing pPC1 148

4.2.14. Isolation of the Maltose Binding Protein-LtnJ Fusion from *E. coli* K12 ER2507 Containing pLS3 150

4.2.15. Cleavage of LtnJ from the Maltose Binding Protein Fusion 151

4.2.16. Preparation of Cell Free Extract from *L. lactis* subsp. *lactis* MG1363 152

4.2.17. Isolation of Lacticin 3147 Mutants from an *L. lactis* Δ*ltnJ* Mutant 152

4.2.18. His_6-LtnJ Activity Assay with Dipeptidyl Substrates using *E. coli* BL21(DE3) containing pPC1 154

4.2.19. LtnJ Activity Assay with Dipeptidyl Substrates using *E. coli* K12 ER2507 containing pLS3 or CFE of *L. lactis* 154

4.2.20. LtnJ Activity Assay with Lacticin 3147 Δ*ltnJ* Mutants using *E. coli* K12 ER2507 containing pLS3 155

4.2.21. LtnJ Activity Assay with Tripeptidyl Substrates using LtnJ from *E. coli* K12 ER2507 containing pLS3 or CFE of *L. lactis* 156

4.2.22. Detection Limits of LCMS 157

4.3. Bacteriocins as Spermicides 159

4.3.1. Bacterial Strains, Plasmids, and Culture Media 159

4.3.2. Antimicrobial Activity Monitoring During Purification 160

4.3.3. Mass Spectrometry 160

4.3.4. Isolation of Nisin A (**16**) 161

4.3.5. Isolation of Lacticin 3147 (**18**, **19**) 162

4.3.6. Collection of Semen Samples 165

4.3.7. Collection and Analysis of Rat Sperm 166

4.3.8. In Vitro Testing of Peptides on Sperm Motility 169

REFERENCES 171

LIST OF TABLES

Table		Page
1.	Classes of antibiotics and their functional targets.	3
2.	Classes of bacteriocins produced by LAB.	5
3.	STI and the causative bacteria.	91
4.	Stability of boar sperm in various solvents.	94
5.	Bacterial strains and plasmids used in LtnJ project.	132
6.	Bacterial strains and plasmids used in bacteriocins as spermicides project.	155

LIST OF FIGURES

Figure		Page
1.	The peptidoglycan layer of bacterial cell walls.	1
2.	Primary structure of subtilosin A.	7
3.	NMR solution structure of subtilosin A.	8
4.	Modified amino acids found in lantibiotics.	10
5.	Primary structure of nisin A.	15
6.	Models for non-targeted pore formation by nisin.	16
7.	The structure of lipid II in most Gram-positive bacteria.	16
8.	A proposed model for Lipid II-mediated pore formation by nisin.	18
9.	Irish kefir grain.	20
10.	Primary structures of lacticin 3147 peptides.	21
11.	Solution NMR structures of lacticin 3147 peptides.	22
12.	Complementary activity of LtnA1 and LtnA2 against *Lactococcus lactis* subsp. *cremoris* HP.	23
13.	A proposed model for Lipid II-mediated pore formation by lacticin 3147.	25
14.	Map of pMRC01.	29
15.	Bacteriocin production and immunity regions of pMRC01.	30
16.	Alignment of LtnJ, SacJ, PenN, Pfam00107, and COG0604.	31
17.	Control of the T7*lac* promoter in the pET System.	33
18.	Coding region of (a) pPC1 and (b) pPC2 and (c) N-terminal tag amino acid sequence for pPC1 and pPC2.	34

19. Western Dot Blots showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing either pPC1 or pPC2 grown at 15 °C, 20 °C, 25 °C, as well as at 30 °C for pPC2. 37

20. Western Blot analysis of the soluble and total protein fractions for *E. coli* BL21(DE3) containing either pPC1 (left) grown at 15 °C, 20 °C, and 25 °C or pPC2 (right) grown at 15 °C, 20 °C, 25 °C, and 30 °C. 37

21. Western Dot Blots showing soluble and insoluble LtnJ production for *E. coli* Origami™ B(DE3) containing either pPC1 (right) or pPC2 (left) grown at different temperatures. 38

22. Western Blot analysis of the soluble and the insoluble fractions for *E. coli* Origami™ B(DE3) containing pPC1. 38

23. Affinity chromatography for the purification of LtnJ from *E. coli* BL21(DE3) cells containing pPC1. 39

24. (a) SDS-PAGE and (b) Western Blot of fractions collected during the purification of LtnJ from *E. coli* BL21(DE3) cells containing pPC1. 40

25. ESMS spectrum (80 eV) of a mixture containing 50 nmol dipeptide **25**, 100 nmol NADH, and 100 μg LtnJ from *E. coli* BL21 (DE3) containing pPC1 in a total volume of 200 μL phosphate buffer. 46

26. (a) Western Dot Blot and (b) Western Blot showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing pLS2 grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. 50

27. (a) Western Dot Blot and (b) Western Blot showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing pLS1B grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. 51

28. (a) Western Dot Blot and (b) Western Blots showing soluble and insoluble LtnJ production for *E. coli* K12 TB1 containing pLS3 grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. 52

29. Coomassie-stained SDS-PAGE gels showing soluble and total protein fractions for *E. coli* BL21(DE3) containing pLS1A grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. 53

30. Coomassie-stained SDS-PAGE gels showing soluble, insoluble and total protein fractions and pilot purifications of LtnJ for *E. coli* K12 TB1, K12 CAG629, K12 UT5600, K12 ER2507, and K12 ER2508 containing pLS3 grown at 37 °C. 55

31. Western Blots showing soluble, insoluble and total protein fractions for *E. coli* K12 TB1, K12 UT5600, K12 ER2507, and K12 ER2508 as well as pilot purifications of LtnJ for *E. coli* K12 UT5600, K12 ER2507, and K12 ER2508 containing pLS3 grown at 37 °C. 56

32. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 1% (w/w) Factor Xa incubated at 25 °C and 4 °C. 58

33. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 2% or 3% (w/w) Factor Xa incubated at 4 °C. 58

34. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 2% or 3% (w/w) Factor Xa incubated at 25 °C. 59

35. ESMS spectrum (80 eV) of control mixture containing 50 nmol dipeptide **25** with 50 nmol dipeptide **35** added, 100 nmol NADH, 100 nmol NADPH, and 100 μg LtnJ from *E. coli* ER2507 containing pLS3 in a total volume of 200 μL Tris buffer. 61

36. ESMS spectrum (80 eV) of a mixture containing 50 nmol dipeptide **25**, 100 nmol NADH, 100 nmol NADPH, and 100 μg LtnJ from *E. coli* ER2507 containing pLS3 in a total volume of 200 μL Tris buffer. 62

37. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **43**, 0.5 nmol tripeptide **58**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=350. (c) LCMS trace of single ion selection for m/z=352. 72

38. ESMS spectum (120 eV) of LC peak (b) from Figure 37 shows presence of tripeptide **43** (m/z=350). 73

39. ESMS spectrum (120 eV) of LC peak (c) from Figure 37 shows presence of tripeptide **58** (m/z=352). 74

40. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **53**, 0.5 nmol tripeptide **59**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=426. (c) LCMS trace of single ion selection for m/z=428. 75

41. ESMS spectum (120 eV) of LC peak (b) from Figure 40 shows presence of tripeptide **53** (m/z=426). 76

42. ESMS spectrum (120 eV) of LC peak (c) from Figure 40 shows presence of tripeptide **59** (m/z=428). 77

43. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **54**, 5 nmol tripeptide **60**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=392. (c) LCMS trace of single ion selection for m/z=394. 78

44. ESMS spectum (120 eV) of LC peak (b) from Figure 43 shows presence of tripeptide **54** (m/z=392). 79

45. ESMS spectrum (120 eV) of LC peak (c) from Figure 43 shows presence of tripeptide **60** (m/z=394). 80

46. (a) LCMS trace of assay reaction containing 50 nmol tripeptide **43**, 100 nmol NADH, 100 nmol NAD^+, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=350. (c) LCMS trace of single ion selection for m/z=352. 81

47. ESMS spectum (120 eV) of LC peak (b) from Figure 46 shows presence of tripeptide **43** (m/z=350). 82

48. ESMS spectrum (120 eV) of LC peak (c) from Figure 46 shows presence of tripeptide **43** (m/z=350) only. 83

49. MALDI-TOF mass spectrum of crude lacticin 3147 mutants prepared using a ZipTip®. 85

50. Alignment of the primary structures of the leader sequences of LtnA1 and LtnA2 shows limited homology. 87

51. The structure of nonoxynol-9. 89

52. Well-plate diffusion assay of nisin and lacticin 3147 against *L. lactis* subsp. *cremoris* HP with and without human semen cryopreservative. 93

53. The effect of 800 μg/mL of nisin, lacticin 3147, and subtilosin A on bovine sperm. 95

54. The effect of 800 μg/mL of nisin, lacticin 3147, and subtilosin A on horse/pony sperm. 96

55. The effect of 200 μg/mL of nisin, lacticin 3147, and subtilosin A on boar sperm. 97

56. The effect of 200 μg/mL of nisin, lacticin 3147, and subtilosin A on rat sperm. 99

57. The minimum inhibitory concentration of lacticin 3147 required to instantly kill bovine, horse/pony, boar, and rat sperm. 100

58. MALDI-TOF mass spectrum of nisin. 158

59. MALDI-TOF mass spectrum of LtnA1. 160

60. MALDI-TOF mass spectrum of LtnA2. 161

LIST OF SCHEMES

Scheme		Page
1.	The formation of lanthionine, methyllanthionine, and D-alanine during the post translation modifications of lantibiotics	11
2.	Formation of D-alanines in lacticin 3147	26
3.	Synthesis of 2,3-didehydroalanine containing dipeptides **24**, **25**, and **26**.	42
4.	Synthesis of D-alanine containing dipeptides **34**, **35**, and **36**.	42
5.	Possible mechanisms for the conversion of 2,3-didehydroalanine to D-alanine by LtnJ using NADH or NADPH as a co-factor.	44
6.	Synthesis of 2,3-didehydroalanine containing tripeptide **43**.	66
7.	Synthesis of 2,3-didehydroalanine containing tripeptides **53** and **54**.	68
8.	Synthesis of D-alanine containing tripeptides **58**, **59**, and **60**.	69

LIST OF ABBREVIATIONS

AA	Amino acid
Abu	α-Aminobutyric acid
ADP	Adenosine diphosphate
AEBSF	4-(2-Aminoethyl)-benzenesulfonyl fluoride•HCl
AMP	Antimicrobial peptide
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
Ala	Alanine
Boc	*tert*-Butoxycarbonyl
BSA	Bovine serum albumin
CFE	Cell free extract
Cys	Cysteine
Da	Daltons
DBU	Diaza(1,3)bicyclo[5.4.0]undecane
DIPCDI	N,N'-diisopropylcarbodiimide
DMAP	4-(Dimethylamino)pyridine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate

DTT	Dithiothreitol
EDC	N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide
EDTA	Ethylenediaminetetraacetic acid
ESMS	Electrospray mass spectrometry
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GlcNAc	N-Acetylglucosamine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GRAS	Generally recognized as safe
GSP	General secretion pathway
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HIV	Human immunodeficiency virus
HOBt	N-Hydroxybenzotriazole
Ile	Isoleucine
IPA	Isopropyl alcohol
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IR	Infrared spectroscopy
LAB	Lactic acid bacteria
LB	Luria Bertani broth
LCMS	Liquid chromatography mass spectrometry

Leu	Leucine
Lys	Lysine
MALDI-TOF	Matrix assisted laser desorbtion ionization – time of flight
MBP	Maltose binding protein
Met	Methionine
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-[4-5-Dimethylthiazol-2-4]-2,5-diphenyltetrazolium bromide
MurNAc	N-Acetylmuramic acid
MWCO	Molecular weight cut-off
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NMM	N-Methylmorpholine
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Phe	Phenylalanine
PMF	Proton motive force
Pro	Proline
PRP	Penicillin resistant *Pneumococcus*
PyBOP®	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate

RNA	Ribonucleic acid
RBS	Ribosome binding sites
RP-HPLC	Reversed phase high performance liquid chromatography
rpm	Revolutions per minute
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
STI	Sexually transmitted infection
TAE	TRIS acetate buffer
TB	Terrific broth
TBE	TRIS-borate-EDTA buffer
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
Thr	Threonine
Trp	Tryptophan
TSBY	Tryptic soy broth yeast extract
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
VRE	Vancomycin resistant enterococci
YT	Yeast extract

CHAPTER 1. INTRODUCTION TO BACTERIOCINS

1.1. The Bacterial Cell Wall and Antibiotics

Nearly all prokaryotes have cell walls external to their plasma membranes. The functions of the cell walls are to maintain the shape of the cell, provide physical protection, and prevent the cell from bursting in a hypoosmotic environment.[1] Bacterial cell walls contain a unique material called peptidoglycan, which consists of polymers of modified sugars cross-linked by short polypeptides that vary from species to species (Figure 1). The cell walls of Gram-positive bacteria contain more peptidoglycan than those of Gram-negative bacteria.[1,2] Gram-negative bacteria also possess an additional outer membrane which contains lipopolysaccharides.

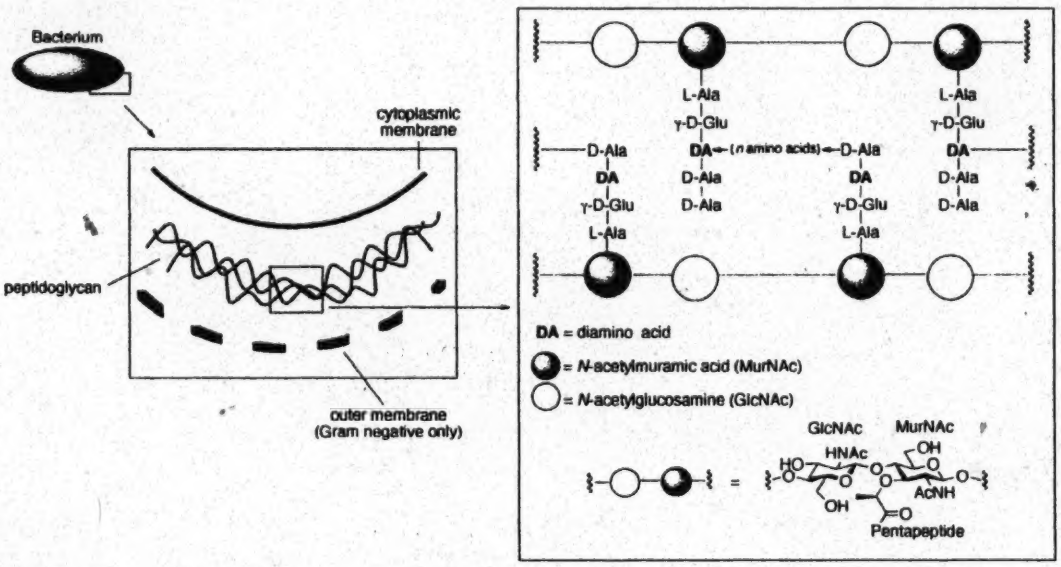

Figure 1. The peptidoglycan layer of bacterial cell walls.[3]

The germ theory of disease, a theory which linked bacteria and other microbes to the causative agent of many ailments, gained wide acceptance in the late 1800s. This began the search for drugs that would kill the disease-causing bacteria, and thus the search for antibiotics. The first such antibiotic, penicillin, was discovered in 1929 by Alexander Fleming.[4] Penicillin inhibits the formation of the cross-links in peptidoglycan and thus prevents the formation of a functional cell wall. As early as 1945, Fleming warned that misuse of penicillin could lead to the selection of resistant forms of bacteria. This foreshadowed a phenomenon now known as antibiotic resistance. Antibiotic resistance can be intrinsic, mutational, or acquired.[5] Examples of bacterial resistance mechanisms include prevention of the antibiotic from entering the cell, inactivation of the antibiotic by the bacterium, and active efflux of the antibiotic from the cell.[6] The need for new antibiotics to combat resistant strains emerged and led to the development of many novel classes of antibiotics (Table 1).

As the number of new antibiotics increased, so did the number of resistant bacterial strains.[7,8,9,10] Eventually a plethora of antibiotic resistant infectious bacteria arose such as methicillin-resistant *Staphylococcus aureus* (MRSA),[10] ceftazidime resistant *Escherichia coli* and *Klebsiella pneumoniae*,[11] streptomycin and rifampicin resistant *Salmonella typhimurium*,[12] and penicillin resistant *Streptococcus pneumoniae*.[13] Vancomycin became known as the drug of last resort, to be used when treatment with other antibiotics failed, as no resistant strains were known. In 1987 vancomycin resistant enterococci (VRE) appeared in hospitals and since then vancomycin resistant *Staphylococcus aureus* has also emerged.[14,15,16] In fact, today there is no antibiotic in clinical use to which resistance

has not developed, and it has been 20 years since a genuinely new class of antibiotics has reached the market.

Table 1. Classes of antibiotics and their functional targets

Antibiotic Class	Example	Functional Target
β-Lactams[17]		Peptidoglycan synthesis
• Penicillins	penicillin, ampicillin, amoxicillin	
• Cephalosporins[18]	cephalexin, cefprozil	
• Carbacephems	loracarbef	
• Carbapenems[19]	ertapenem, meropenem	
• Monobactams	aztreonam	
Glycopeptides[20]	vancomycin, teicoplanin	
Polypeptides[21]	polymyxin B, bacitracin	
Macrolides[22]	erythrmycin, clarithromycin	Protein synthesis
Lincosamides[7]	lincomycin, clindamycin	
Tetracyclines[23]	tetracycline, doxycycline	
Aminoglycosides[24]	gentamicin, streptomycin	
Oxazolidinones[25]	linezolid	
Rifamycins[26,27]	rifampin	
Streptogramins[28]	streptogramin A	
Sulfonamides[29]	co-tremoxazole, trimethoprim	Folate synthesis
Nitrofurans[30]	nitrofurantoin	RNA metabolism
Quinolones[31,32,33]	levofloxacin, ciprofloxacin	DNA replication/transcription

1.2. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides from bacteria that are typically active against bacteria closely related to the producer strain. These peptides are potent antibiotics and it has been proposed that 99% of bacteria produce at least one bacteriocin.[34] To protect themselves from their own product, bacteriocin-producing organisms typically rely on dedicated peptides or proteins called immunity proteins.[35,36] Lactic acid bacteria (LAB) are particularly fruitful in their production of bacteriocins and several classes have been proposed by Klaenhammer (Table 2).[35]

Class I includes the lantibiotics, antimicrobial peptides containing the unusual post-translationally modified lanthionine and/or β-methyllanthionine residues. This class can be further subdivided into type A and type B. Type A are elongated peptides that kill target cells by forming pores in their cell membranes. In contrast, type B lantibiotics are globular and inhibit enzyme functions by binding to membrane lipids.

Class II bacteriocins are small, cationic, <10 kDa, heat stable non-lanthionine containing peptides. They are organized into three subgroups; IIa are termed YGNGV(X)C-type as they all contain a conserved amino-terminal sequence (YGNGV(X)C), IIb are two-peptide bacteriocins, and IIc are small heat stable bacteriocins that do not fall in the other two categories.

Table 2. Classes of bacteriocins produced by LAB

Class	Characteristics	Subclasses and Examples
I. Lantibiotics	- Small (<5 kDa), ribosomally produced peptides - Contain intramolecular thioether rings formed by lanthionine and β-methyllanthionine - Highly posttranslationally modified	**Type A.** Elongated, amphipathic, cationic, pore-forming Eg. nisin A[37] **Type B.** Globular, anionic or no net charge, enzymatic inhibition Eg. mersacidin[38]
II. Nonlantibiotics	- Small (<10 kDa), ribosomally produced, heat stable (100 °C to 121 °C) peptides - Minimal post translational modification involving cleavage at a double glycine site to release the mature peptide from its prepeptide - Cationic	**IIa.** Conserved sequence YGNGV(X)C, *Listeria* active, pore-forming Eg. pediocin PA-1[37,39] leucocin A[37] **IIb.** Two-peptide bacteriocins, pore-forming Eg. plantaricin EF[40] **IIc.** Other (no YGNGV(X)C sequence) one-peptide bacteriocins, some are sec-dependent[a] Eg. lactococcin 972[41]
III. Non lantibiotics	- Large (>30 kDa), heat labile peptides	Eg. helveticin J[42]
IV.[b]	- Bacteriocins requiring lipid or carbohydrate moieties	Eg. lactocin 27[43]

[a] Bacteriocins dependent on export using the translocase general secretory (sec) pathway (GSP)
[b] Currently this class is undefined

Class III bacteriocins are large, >30 kDa, heat labile proteins. Thus far this class is less well characterized than either class I or class II and only a handful of class III bacteriocins have been isolated from LAB.

Class IV bacteriocins are loosely defined as proteins that are associated with other lipid or carbohydrate moieties, which are required in order to elicit their antimicrobial activity. Members of this class have not been adequately characterized at the biochemical level.

Due to the emergence of antibiotic resistance, the use of bacteriocins as antimicrobial agents is currently being explored. For example, staphylococcins, bacteriocins produced by staphylococci, are being evaluated for their use against multiresistant *Staphylococcus aureus* and coagulase-negative staphylococci, both of which are involved in human infections.[44] Furthermore, the *in vivo* activity of mersacidin, a type B lantibiotic, against MRSA colonizing the nasal epithelia of mice shows promising results.[45] In addition to medical applications, bacteriocins are finding use in skin therapy as well as various food applications. For instance, a bacteriocin produced by *Lactococcus* sp. HY 449 has been evaluated for its use as a cosmetic ingredient.[46] This bacteriocin is active against the skin-inflammatory bacteria *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Propionibacterium acnes*. Bacteriocins are also being used in probiotics and as food preservatives in many types of foods including vegetables, fruit juices, cheeses, and meats.[47,48,49,50,51,52,53,54]

1.2.1. Subtilosin A

Subtilosin A (**1**) is a bacteriocin produced by *Bacillus subtilis* JH642, a spore-forming, soil bacterium found in many oriental fermented foods. This bacteriocin was first isolated in 1985 from a Chinese fermented soybean culture.[55] Unlike class II bacteriocins in LAB where a leader peptide is cleaved after a double Gly-Gly motif, cleavage of the subtilosin A leader peptide occurs between Asn 1 and Glu -1. In addition, subtilosin A is highly post-translationally modified yielding an amide bond between the N-terminal asparagine and the C-terminal glycine as well as three unusual sulfur-α-carbon bridges (Figure 2).

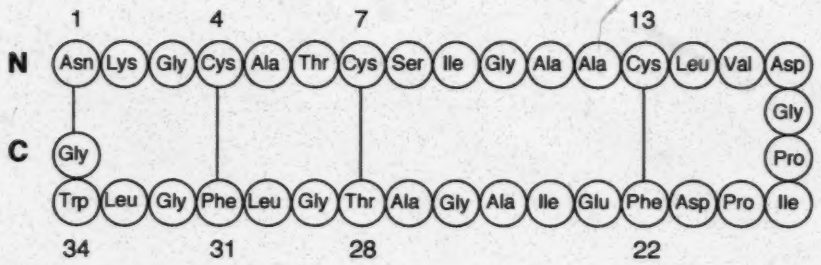

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Figure 2. Primary structure of subtilosin A. Solid lines indicate the positions of the post-translationally formed linkages.

Subtilosin A is *Listeria* active and the presence of the sulfur-α-carbon bridges are necessary for biological activity. Uncyclized subtilosin A (asparagine 1 and glycine 25 linkage) with the sulfur bridges still intact shows only a slight reduction in activity.[56]

Previously, Kawulka *et al.* elucidated the primary and three dimensional structure of subtilosin A by solution NMR, revealing the stereochemistry of the sulfur-α-carbon bridges to be L-Phe22, D-Thr28, D-Phe31 (Figure 3).[57] At present, the mechanism of antimicrobial action for subtilosin A is unknown, although it is speculated to be receptor-mediated as the peptide has an overall negative charge at neutral pH.[56] Subtilosin A represents a new class of bacteriocins as it is the first ribosomally synthesized peptide to contain the unusual sulfur-α-carbon bridges.

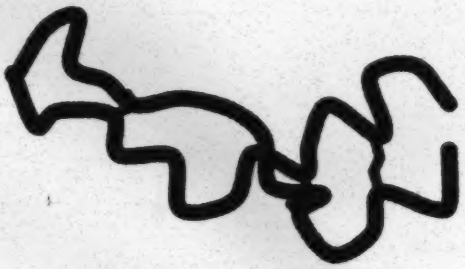


Figure 3. NMR solution structure of subtilosin A.

1.3. Lantibiotics

Lantibiotics are bacteriocins that undergo extensive posttranslational modifications. The name lantibiotics was introduced in 1988 to include peptides that contain lanthionine (**2**) and β-methyllanthionine (**3**) residues (Figure 4).[58] These cyclic residues are derived from the Michael addition of cysteine sulfurs on dehydrated serine and threonine residues (Scheme 1). Many lantibiotics possess additional modified amino acids such as 2,3-dehydroalanine (**4**) (Dha), 2,3-dehydrobutyrine (**5**) (Dhb), as well as the less common modifications N,N-dimethylalanine (**6**), lysinoalanine (**7**), *S*-aminovinyl-D-methylcysteine (**8**), β-hydroxy-aspartate (**9**), D-alanine (**10**), *S*-aminovinyl-D-cysteine (**11**), 2-oxopropionate (**12**), 2-oxobutyrate (**13**), *allo* isoleucine (**14**), and 2-hydroxy-propionate (**15**) (Figure 4).[59]

As mentioned in section 1.2, lantibiotics have been subclassed into type A, elongated amphipathic peptides, and type B, globular peptides. Type A lantibiotics are further subdivided based on the modification enzymes involved in their biosynthesis.[59] Type AI peptides, such as nisin A and epidermin, use two distinct enzymes (LanB and LanC) in the formation of lanthionine and β-methyllanthionine residues.[60,61] LanB enzymes perform the dehydration of serines and threonines while LanC enzymes catalyze the cyclization reactions. Type AII lantibiotics, such as lacticin 481 and salivaricin A, use a single enzyme (LanM) to perform both the dehydration and cyclization reactions.[62,63]

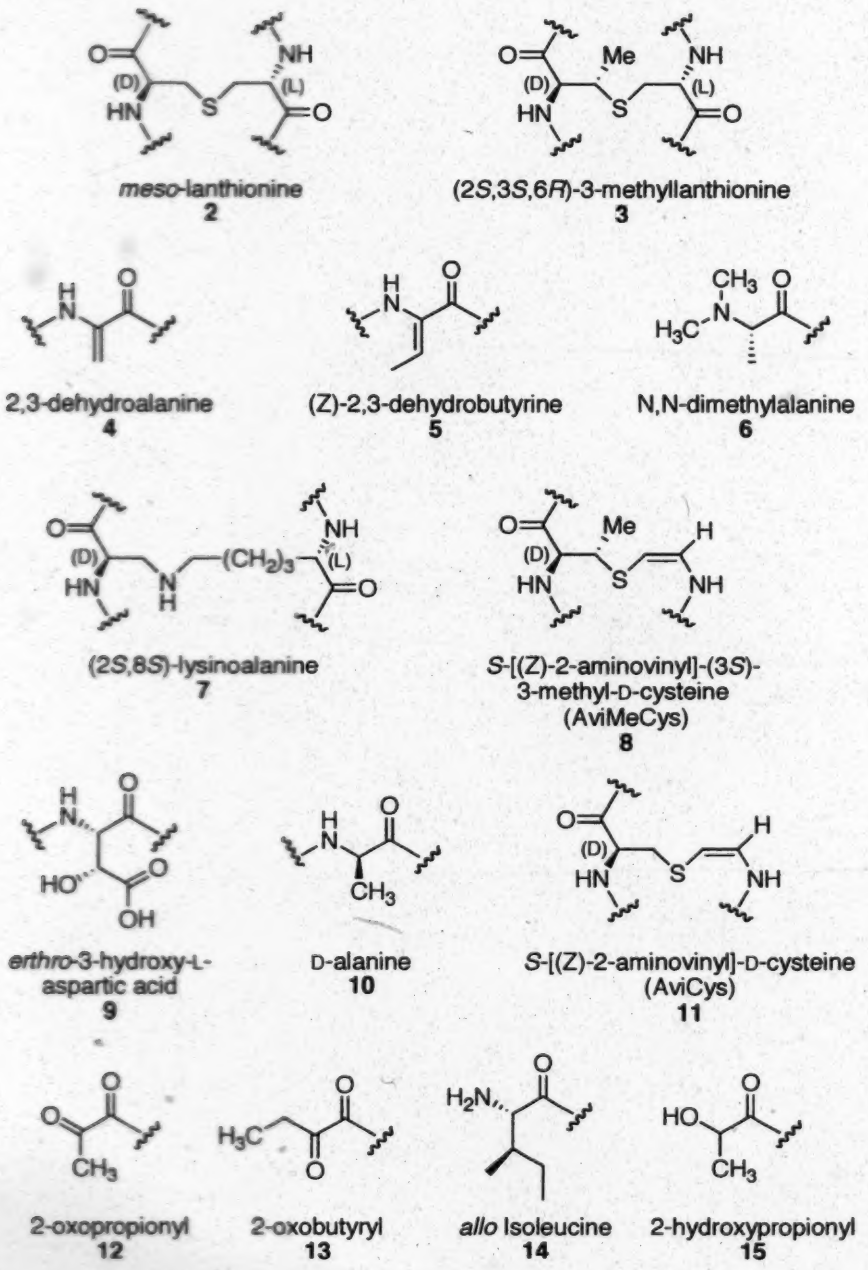
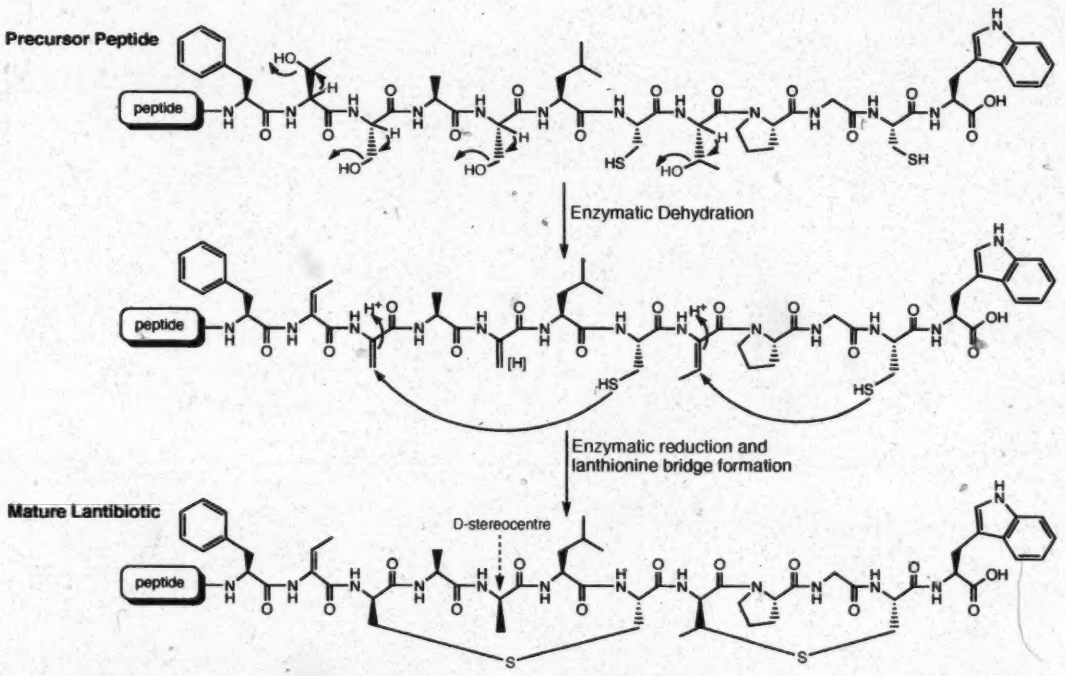


Figure 4. Modified amino acids found in lantibiotics.



Scheme 1. The formation of lanthionine, methyllanthionine, and D-alanine during the post translation modifications of lantibiotics.

All lantibiotic precursor peptides are ribosomally produced such that they contain 23-59 extra amino acids at their N-termini that extend beyond the residues found in the mature lantibiotic.[59] These extra residues correspond to leader peptides and are not post translationally modified even though in many cases they contain serine and threonine amino acids. The exact function of the leader peptide has not been determined but plausible roles may include a signal for bacteriocin export, immunity for the producer strain by keeping the peptide inactive, or a recognition scaffold for modifying enzymes.[64,65] Supporting evidence for each proposed function has been found for various lantibiotics. For example, nonlantibiotic peptides can be exported by the nisin NisT transporter if they contain the nisin leader sequence.[66] In addition, some modification

enzymes have been shown to recognize the leader peptide of the bacteriocins that they modify and cannot modify their bacteriocin precursor if its leader peptide has been removed. For example, the LanM-type enzyme LctM responsible for the dehydration and cyclization reactions of lacticin 481 has been shown to require at least part of the lacticin 481 leader sequence.[67] Mutant propeptides containing three or eight amino acid deletions in the leader sequence were fully processed by LctM, yet the propeptide containing none of the leader sequence was not processed. Furthermore, the nisin cyclization enzyme, NisC, also requires the nisin leader sequence in order to convert the dehydrated nisin into its bioactive product.[68] In contrast, at least one modification enzyme has been shown to not require the leader sequence. EpiD, a modification enzyme involved in the formation of AviCys (**11**) (Figure 4) in the lantibiotic epidermin, is able to process peptides, both *in vitro* and *in vivo*, that do not contain the leader sequence.[69,70,71] Lastly, many fully processed lantibiotic peptides are not biologically active if the leader sequence is still attached, indicating that the leader sequence may serve a protective role.[68,72,73]

In order to elicit their killing, bacteriocins must be exported from the cell, where they are synthesized and modified, into the extracellular space. This is done using an ATP binding cassette (ABC) transporter. In most type AI lantibiotics, the leader peptide is cleaved by a separate serine-like protease (LanP) after export by the transporter (LanT).[74,75] In contrast, type AII lantibiotics do not have an equivalent LanP enzyme and instead, it has been proposed that the LanT enzyme processes the leader peptide concomitant with bacteriocin export.[63,76] The gene clusters for type B lantibiotics have not generated as much attention and study as those for type A lantibiotics. The gene

cluster for mersacidin, a type B lantibiotic, contains a *lant* component similar to that of type AII lantibiotics.[77]

Recently, lantibiotics have received much attention due to their multiple applications. Perhaps the largest area of lantibiotic research is centered on food preservation. Many lantibiotics are produced by food-grade organisms such as lactococci, carnobacteria, and lactobacilli,[78] and inhibit the food borne pathogens *Listeria monocytogenes* and *Clostridium botulinum*.[79,80] This makes their development as preservatives in processed foods, such as packaged meats and dairy products, extremely attractive. Lantibiotics are also being investigated for their potential therapeutic use. For example, gallidermin, epidermin, and lacticin 3147 are particularly effective against the pathogenic organism *Propionibacterium acnes*, one of the causative bacteria of acne.[60,81] In addition, mersacidin and lacticin 3147 are active against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE), and actagardine is active against streptococci.[46,82,83,84] Furthermore, cinnamycin and duramycin are both inhibitors of phospholipase A2, an enzyme involved in the immune system.[85]

1.3.1. Nisin

The most well studied lantibiotic, nisin, is a member of the type AI peptides. Nisin A (**16**) was discovered in 1928[86] yet Gross and Morell did not elucidate its structure until 1971 (Figure 5).[87] The bacteriocin contains one lanthionine bridge, four β-methyllanthionine bridges, and the unusual amino acids 2,3-dehydroalanine and 2,3-dehydrobutyrine. Since the discovery of nisin A, several naturally occurring variants have been found including nisin Z,[88] nisin Q,[89] and very recently, nisin U.[90] Nisin Z and nisin Q differ from nisin A by one and four amino acids, respectively. Nisin U differs by nine amino acids and has a C-terminus truncated by three amino acids as compared to nisin A. Nisin A is produced by the food grade bacteria *Lactococcus lactis* subsp. *lactis* and has received GRAS (generally recognized as safe) status by the United States of America Food and Drug Administration. It is currently used as a food preservative in over 80 countries worldwide and is the active ingredient of the commercial products Nisaplin® and Novasin™ used in food preservation. This bacteriocin prevents the spoilage of food by *Clostridium*, *Staphylococcus*, *Bacillus*, and *Listeria*.[91] Nisin is also the active ingredient in two other commercial products, Consept® and Wipe-Out®, used to prevent bovine mastitis, a bacterial inflammation of the udder in dairy cattle. In addition, a number of patents have been filed for the use of nisin in chewing gum as the active ingredient against tooth decay.[92,93] Another recently discovered application of nisin is its use as a potential spermicide as it inhibits the motility of rat, rabbit, monkey, and human sperm.[94,95]

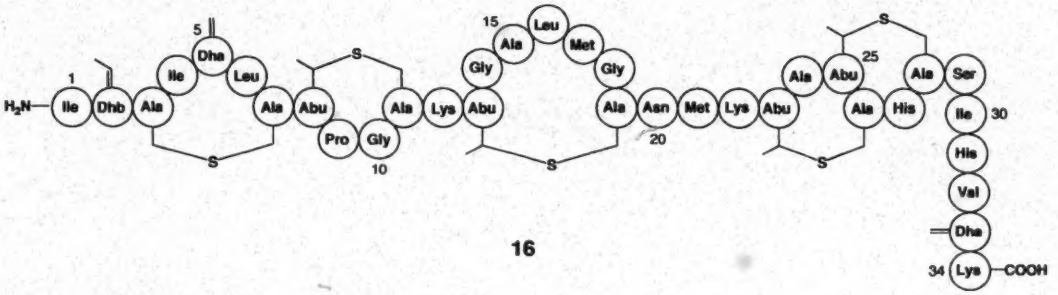


Figure 5. Primary structure of Nisin A.

Because of its importance in food preservation, much research has been done to determine nisin's mechanism of bacterial killing. Nisin's nanomolar minimum inhibitory concentration (MIC) against various Gram-positive bacteria is attributed to several methods of killing; pore formation, disruption of cell wall biosynthesis, prevention of the outgrowth of spores, and induction of autolysis.[96,97,98,99]

The formation of pores by nisin in bacterial cell membranes results in the rapid efflux of metabolites and the dissipation of the membrane potential due to the loss of potassium and inorganic phosphate. This leads to the hydrolysis of cytoplasmic ATP as the cell attempts to re-accumulate these ions and the collapse of the pH gradient causing cell death. The pore forming ability of nisin begins with the highly positively charged C-terminus of the peptide interacting with the anionic membrane surface of the target bacterial cell. Two models have been proposed for the pore formation, the barrel-stave and the wedge model (Figure 6).[100,101] In the barrel-stave model, the nisin molecules assemble into pre-aggegates and then, at a certain membrane potential, form pores in which the peptides become perpendicular with respect to the membrane.[100] The peptide

adopts a membrane spanning orientation where the hydrophobic surface of the peptide is exposed to the core of the membrane while the hydrophilic side forms the aqueous channel. In the wedge model, the nisin molecules bind parallel to the membrane and produce local strain. This strain leads to bending of the membrane and allows the lipid molecules to make the pore along with nisin.[101] The orientation of the nisin molecules with respect to the lipid headgroups remains unchanged. In both models the lifetime of these nisin-induced pores is short, milliseconds, with a diameter of 0.2-2 nm.[96]

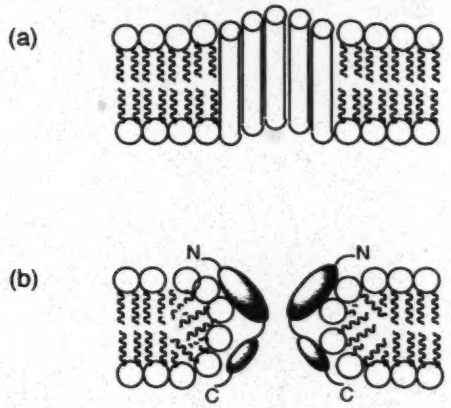


Figure 6. Models for non-targeted pore formation by nisin. (a) Barrel-stave pore. (b) Wedge pore.

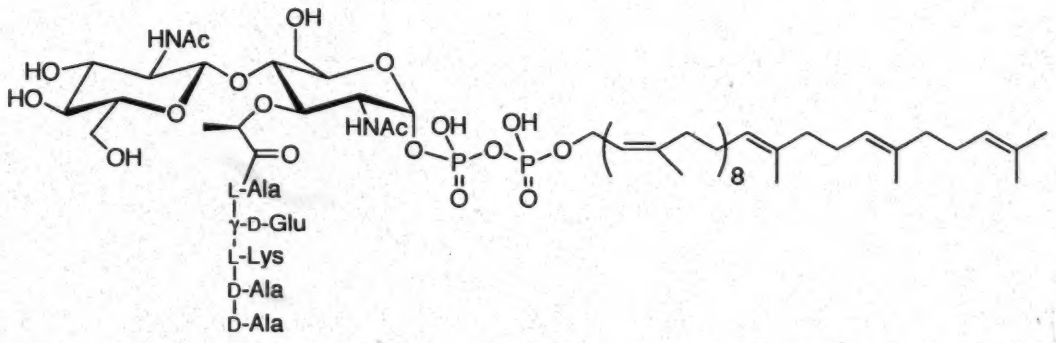


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Figure 7. The structure of lipid II in most Gram-positive bacteria.

Nisin can also form longer-lived pores (several seconds) with a 2 nm diameter by binding to the peptidoglycan precursor lipid II (**17**) (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc) (Figure 7), which acts as a docking molecule for this bacteriocin.[96] The peptidoglycan head group of lipid II, MurNAc-(pentapeptide)-GlcNAc, serves as the basic building block for bacterial cell walls while the pyrophosphate-undecaprenyl lipid tail functions to carry the peptidoglycan moiety from the cytoplasm to the extracellular space. The N-terminus of nisin forms a cage-like structure and binds the pyrophosphate moiety, MurNAc, and the first isoprene unit of lipid II.[102] This binding results in a change in the orientation of nisin from parallel to perpendicular with respect to the membrane surface.[103] Multiple molecules of the nisin-lipid II complex aggregate together and a pore is formed consisting of four lipid II and eight nisin molecules.[102,104] A model that is consistent with all the experimental evidence has been proposed for lipid II-mediated pore formation by nisin and is pictured in Figure 8.[59,105]

In addition to forming pores, the binding of nisin to lipid II results in the disruption of cell wall biosynthesis. When lipid II is bound to nisin it is no longer physically available for incorporation into the peptidoglycan layer of the cell wall. In fact, nisin mutants that were still able to bind to lipid II yet were unable to form pores resulted in only slightly reduced activity *in vivo*.[97] Thus, nisin's interaction with lipid II leads to two killing mechanisms in one molecule, resulting in its nanomolar activity against numerous Gram-positive bacterial strains.

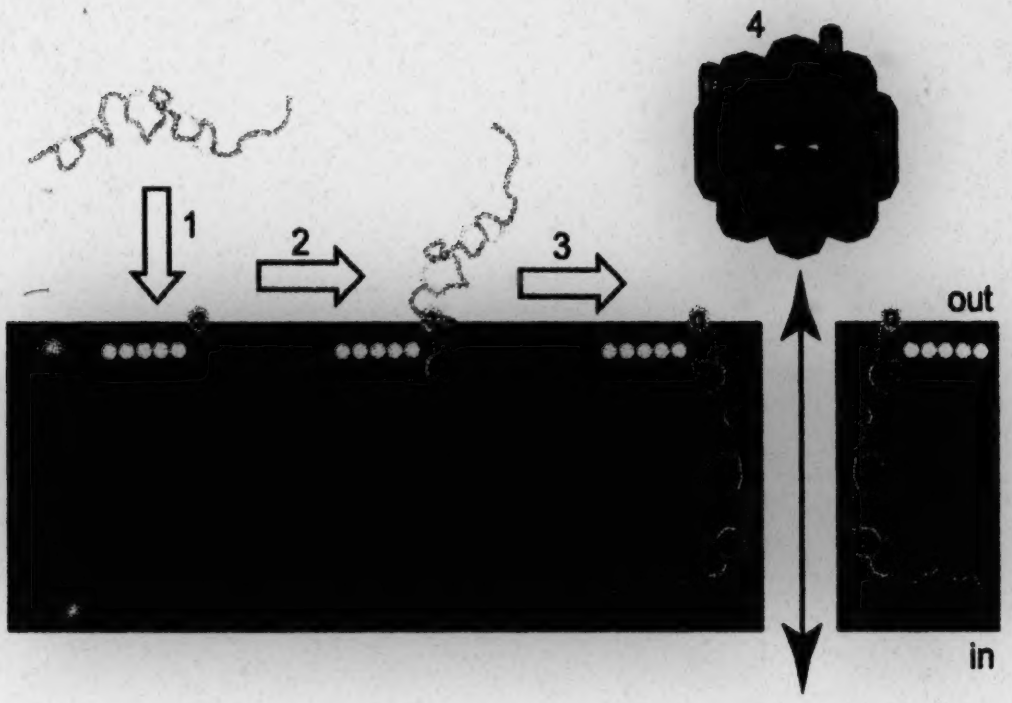


Figure 8. A proposed model for lipid II-mediated pore formation by nisin. (1) The cationic nature of nisin facilitates its interaction with the bacterial plasma membrane. (2) Nisin binds to lipid II via two of its N-terminal rings. (3) The transmembrane orientation of nisin results in the formation of a pore. (4) The 2 nm diameter pore consists of eight nisin molecules and four lipid II molecules. (Reproduced with permission from Molecular Microbiology, Breukink, 2006).[105]

The extensive use of nisin in food preservation is partially attributed to its ability to inhibit the germination of spores from *Bacillus* and *Clostridium* species. Nisin interferes with the modification of sulfhydryl groups on the exterior of spores, a crucial step for spore outgrowth.[98] This constitutes yet a third mechanism of biological activity for this lantibiotic peptide.

Lastly, nisin is able to displace the cell wall lytic enzymes from their compartments resulting in premature and uncontrollable lytic activity.[99] The result is lethal cell lysis. This mechanism of antimicrobial action is also independent of lipid II binding and contributes to the overall bacteriocidal activity.

In addition to Gram-positive species, nisin shows biological activity towards some Gram-negative organisms, albeit at much higher MIC values. In order to elicit its antimicrobial activity, nisin must interact with the cytoplasmic membrane. This membrane is protected in Gram-negative bacteria by the outer membrane, giving rise to the observed higher MIC values. For example, nisin A shows an MIC of 10 μM for *Pseudomonas aeruginosa* ATCC 27853.[106] Many Gram-negative bacteria do not show any sensitivity to nisin unless the integrity of the outer membrane is disrupted.[107] Disruption of this outer membrane gives nisin access to lipid II in the cytoplasmic membrane. This has been demonstrated for sublethally injured *Salmonella*, *Enterobacter*, *Shigella*, *Escherichia*, and *Citrobacter* species where the cells were treated with EDTA in combination with nisin (15 μM).[108] EDTA complexes the magnesium ions required for the stability of the outer lipopolysaccharide layer, rendering it more susceptible to nisin. Surprisingly, nisin Z shows an MIC of 600 nM for *Escherichia coli* and shows no antimicrobial activity against *E. coli* in the presence of NaCl.[108] This is an unusually low MIC value for nisin against a Gram-negative organism and implies that the mechanism of antibacterial activity is a high-salt-sensitive, cationic amino acid-mediated mechanism as opposed to the lipid II-mediated mechanism found for Gram-positive organisms.

1.3.2. Lacticin 3147

Lacticin 3147 is a two-component lantibiotic produced by *Lactococcus lactis* consisting of the two peptides LtnA1 **18** and LtnA2 **19**. The bacteriocin producing organism was first isolated by Ross and Hill from an Irish kefir grain used in a fermented, probiotic milk beverage called Kefir (Figure 9).[109] Kefir grains are comprised of protein, lipids, and a soluble polysaccharide (Kefiran), and are rich in lactobacilli, lactococci, and yeasts.[110]

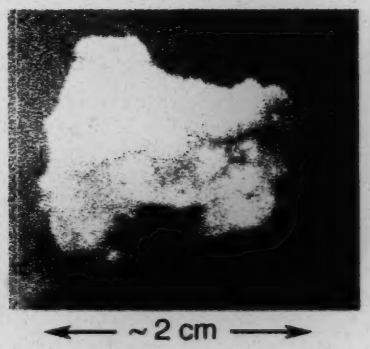
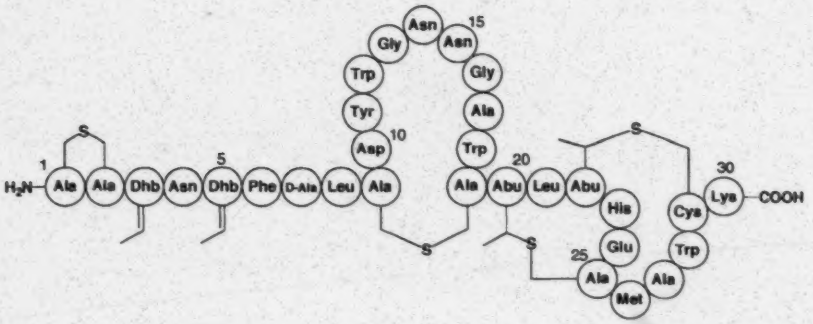


Figure 9. Irish kefir grain.

Martin *et al.* have elucidated the structures of both the LtnA1 (**18**) and LtnA2 (**19**) peptides (Figure 10).[111] Both peptides contain lanthionine and β-methyllanthionine bridges as well as 2,3-dehydrobutyrine amino acids. Interestingly, LtnA1 contains one D-alanine residue whereas LtnA2 contains two of these uncommon amino acids. Furthermore, LtnA2 contains a 2-oxybutryl moiety resulting from the spontaneous deamination of an N-terminal 2,3-dehydrobutyrine. The solution NMR structures of these peptides reveal even more information about their lantibiotic classification as well

as their possible mode of antimicrobial action (Figure 11). LtnA1 exhibits a globular structure, resembling type B lantibiotics while LtnA2 has an elongated, screw-shaped structure, resembling type A lantibiotics.

LtnA1



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LtnA2

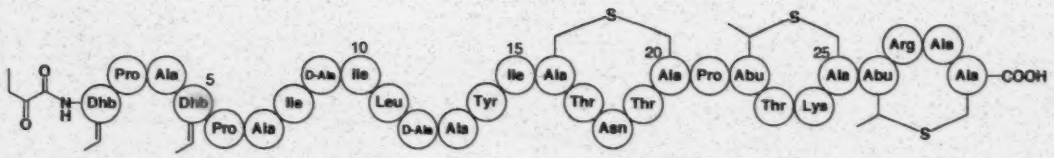

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Figure 10. Primary structures of lacticin 3147 peptides.

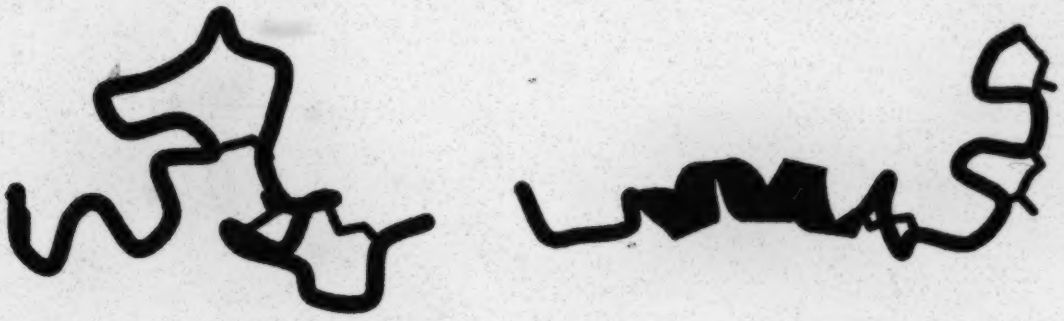


Figure 11. Solution NMR structures of lacticin 3147 peptides.

Individually, the lacticin 3147 peptides have weak activity, in the mM range, but synergistically in a 1:1 ratio, they display strong antibacterial activity in the nM range (Figure 12).[112] There has been widespread interest in lacticin 3147 due to its high activity against methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), and penicillin resistant *Pneumococcus* (PRP).[85] Furthermore, lacticin 3147 is active against the food spoilage organism *Listeria monocytogenes* and thus live lacticin 3147-producing cultures have been used in cheese production to extend the shelf-life of these products.[113] The use of lacticin 3147 in a teat seal to prevent bovine mastitis caused by *Staphylococcus aureus* and *Streptococcus dysgalactiae* also shows promising results.[114,115]

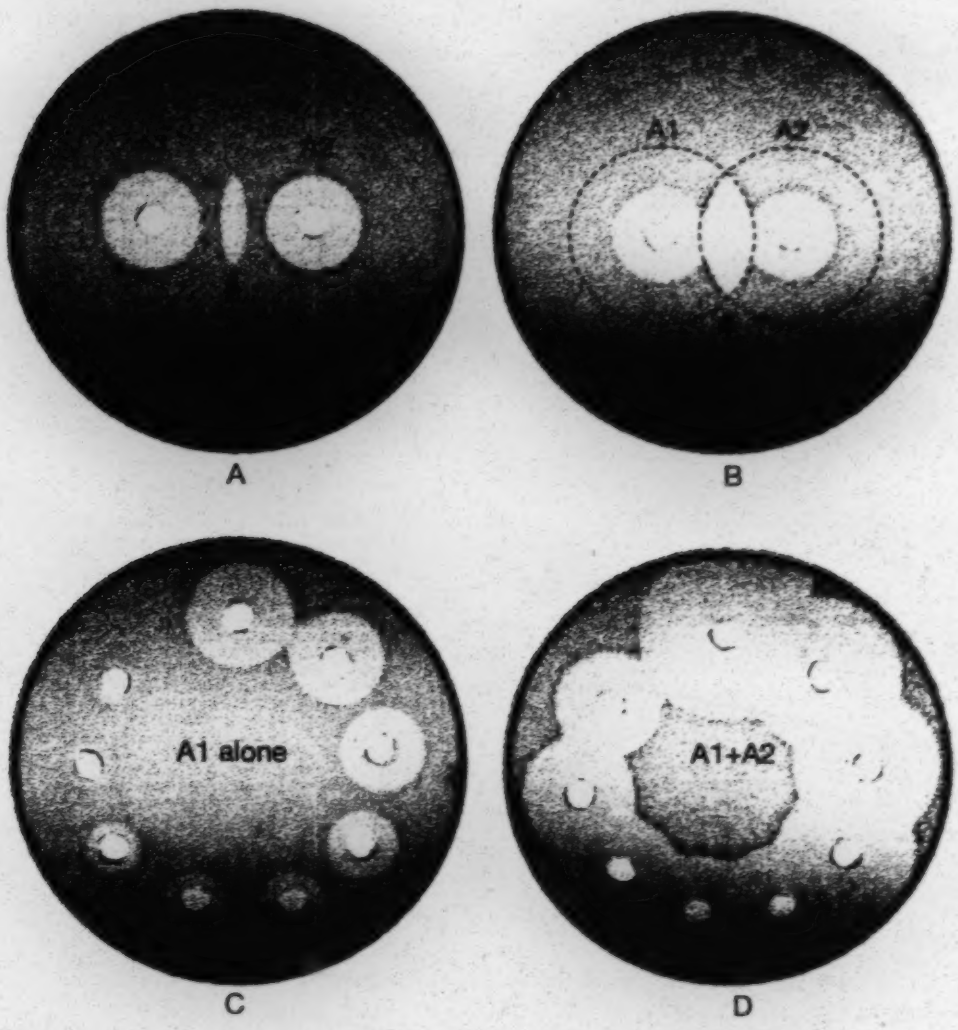


Figure 10. Complementary activity of LtnA1 and LtnA2 against *Lactococcus lactis* subsp. *cremoris* HP. The first two assays (A and B) show the synergy of the peptides as they are spotted side-by-side to form an inhibition zone wherein the two peptides have diffused together. The third assay (C) shows a serial, two-fold, dilution of the A1 peptide alone and the fourth assay (D) contains the same serial dilution of the A1 peptide with the addition of a matching quantity of the A2 peptide in each well. (Reproduced from Martin, N.I., University of Alberta PhD thesis, 2004)[3]

Similar to nisin, lacticin 3147 forms pores in its target bacterial cell membranes using lipid II as a docking molecule. These pores are smaller, 0.6 nm, than those formed by nisin, 2 nm, and only allow the efflux of potassium ions and phosphate. Very recently, Wiedemann *et al.* have proposed a model for the mechanism of antimicrobial action of lacticin 3147 (Figure 13).[116] As with most other type B lantibiotics, LtnA1 carries no net charge, thus its interaction with the cell membrane is strictly governed by its binding with the MurNAc-pyrophosphate moiety of lipid II. This binding most likely induces a conformation in LtnA1 which aids in the subsequent binding of LtnA2, giving rise to a two peptide:lipid II complex. Wiedemann *et al.* speculate that the elongated structure and cationic nature of the C-terminus of LtnA2 facilitates its ability to assume a transmembrane conformation following complex formation, leaving the N-terminus bound to the LtnA1:lipid II complex. The formation of a defined pore is the result with a 1:1:1 stoichiometry (LtnA1:LtnA2:lipid II). Breukink has hypothesized that the pore is made up for four two peptide:lipid II complexes as depicted in Figure 13.[105]

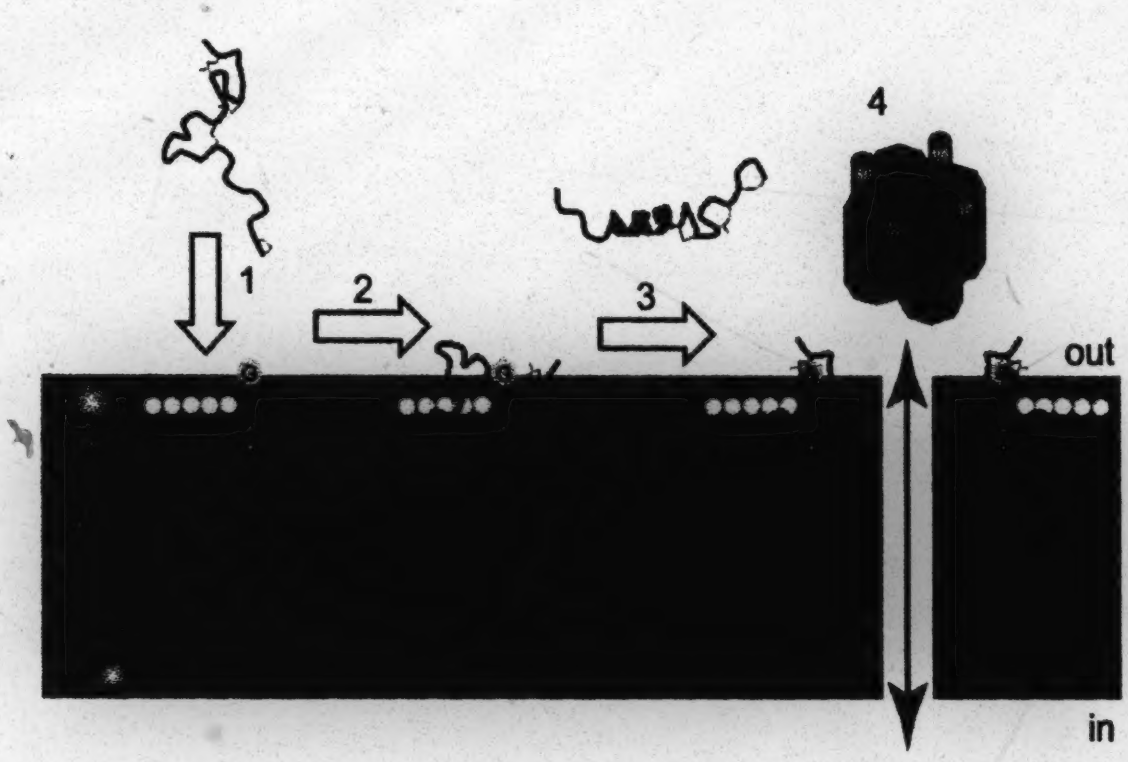
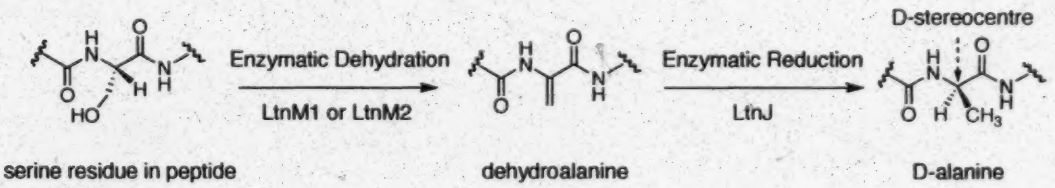


Figure 13. A proposed model for Lipid II-mediated pore formation by lacticin 3147. (1) LtnA1 binds to lipid II. (2) The LtnA1-lipid II complex inserts into the bacterial plasma membrane, which induces a conformational change in LtnA1. (3) LtnA2 binds to the LtnA1-lipid II complex and a pore is formed. (4) The 0.6 nm diameter pore may have a stoichiometry of 4:4:4 LtnA1:lipid II:LtnA2. (Reproduced with permission from Molecular Microbiology, Breukink, 2006).[105]

Because LtnA1 uses lipid II as a docking molecule, this peptide is also able to inhibit peptidoglycan synthesis in a similar fashion as nisin. Moreover, lacticin 3147 is a potent pore forming bacteriocin even in the absence of lipid II (***unpublished results, personal communication with Weidemann***). Cotter *et al.* have shown that the D-alanines in lacticin 3147 are absolutely essential for full biological activity.[117] Replacement of the

D-alanine centers in LtnA2 with L-alanine resulted in a four-fold reduction in activity for a single D to L conversion and a 16-fold loss in activity with replacement of both D-centers. For both LtnA1 and LtnA2, replacement with either 2,3-dehydroalanine, 2,3-dehydrobutyrine, glycine, or valine also resulted in a loss of biological activity, although replacement of D-alanine with non-chiral amino acids yielded less significant losses. The exact function of these D-amino acids in the mechanism of pore formation is unknown, but the chirality of theses residues may be required for stereospecific interactions.

The formation of the D-alanines in lacticin 3147 begins with the dehydration of ribosomally synthesized serines to yield 2,3-dehydroalanine. This dehydration is accomplished by LtnM1 in LtnA1 and LtnM2 in LtnA2.[118] The LtnM enzymes are also responsible for the lanthionine and β-methyllanthionine formation via cysteinyl attack on select dehydrated residues. In both lacticin peptides, 2,3-dehydroalanine residues that are not incorporated into cyclic thiols are further modified by LtnJ to give rise to the corresponding D-alanine (Scheme 2).[119]



Scheme 2. Formation of D-alanines in lacticin 3147.

1.4. Objectives

The need for new classes of antibiotics is on the rise due to the emergence of antibiotic resistant strains. A new source of antimicrobial agents may simply come from the bacteria themselves. Bacteria secrete antimicrobial peptides to protect their ecological niches. Many bacteriocins, such as lantibiotics, are comprised of unusual amino acids that are essential for biological activity. D-Alanine is one such amino acid and is found in the two-component bacteriocin lacticin 3147. Imitating nature's design, it may be advantageous to incorporate D-amino acids into other known biologically active peptides in order to increase their functionality. In addition to potentially increasing the biological activity of the modified peptide, D-amino acids would also confer improved chemical stability to proteolytic enzymes. At present, chemical synthesis is the only way to integrate D-amino acids into peptidic structures. An attractive alternative is to use enzymatic methods to create D-residues in a ribosomally synthesized peptide. The recent identification of the enzyme LtnJ, responsible for D-alanine formation in lacticin 3147, presents a potential opportunity for the formation of enzymatically synthesized D-amino acids. To date, the isolation and purification of LtnJ has not been attempted and no known activity assay exists for this enzyme.

In this thesis, attempts to isolate, purify, and develop an *in vitro* activity assay for LtnJ are described. Various *E. coli* over-expression strains were constructed for the isolation of LtnJ including several hexahistidine tagged systems and a maltose-binding protein fusion. The development of an activity assay for LtnJ was explored using several

small, chemically synthesized peptides (three dipeptides and three tripeptides) as LtnJ substrates and various assay conditions.

Further to their antimicrobial properties, some bacteriocins, such as nisin, have demonstrated spermicidal activity.[94,95] This is an interesting and valuable discovery as there is a great need for products that provide dual protection against sexually transmitted infections and pregnancy. Currently, most vaginal contraceptive products contain nonoxynol-9 as a spermicidal ingredient. The trouble with this membrane surfactant is that it is rather non-specific to sperm and exerts a detergent-type cytotoxic effect on vaginal cells. With recurrent use, nonoxynol-9 can actually increase the contraction of sexually transmitted infections in the female user. Thus the development of spermicidal microbicides that are non-toxic to vaginal cell membranes is desirable. Antimicrobial peptides are a group of compounds that may be able to fill this role.

The second project described in this thesis includes the testing of the bacteriocins nisin, lacticin 3147, and subtilosin A as spermicidal agents. Animal sperm from bulls, horses/ponies, boars, and rats were tested.

CHAPTER 2. LTNJ, A MODIFICATION ENZYME IN LACTICIN 3147

2.1. The Bacteriocin Producing Plasmid pMRC01 from *L. lactis* subsp. *lactis* DPC3147

The genetic sequence that encodes bacteriocin production and immunity in *Lactococcus lactis* subsp. *lactis* DPC3147 is found on the 60 kb plasmid pMRC01.[119] The plasmid is organized into three approximately 20 kb functional regions encoding genes for conjugal transfer, bacteriocin production, and bacteriophage resistance and plasmid maintenance with insertion sequences (IS) for each of these regions (Figure 14).

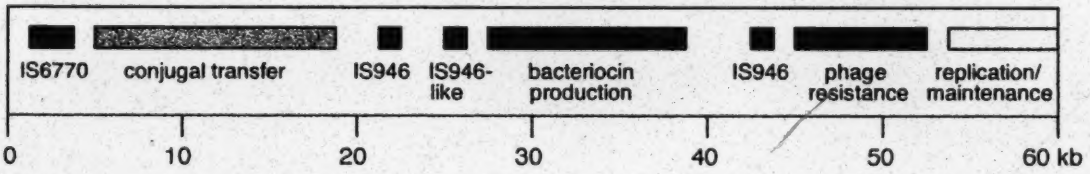


Figure 14. Map of pMRC01.

The six genes responsible for lacticin 3147 production are arranged in an operon that is divergently upstream from a four gene operon (*ltnE*, *ltnF*, *ltnI*, *ltnR*) that encodes for bacteriocin immunity (Figure 15). The genes involved in bacteriocin production are *ltnA1* and *ltnA2*, encoding for the lacticin 3147 prepropeptides, *ltnM1* and *ltnM2*, encoding for post-translational dehydration and lanthionine ring formation, *ltnT*, encoding for bacteriocin export, and *ltnJ*, encoding for post-translational D-alanine formation.

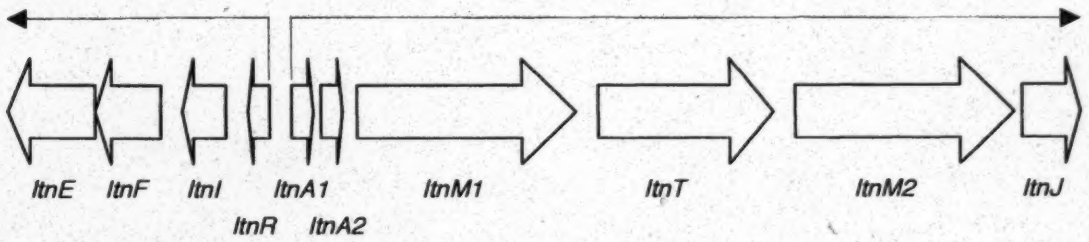


Figure 15. Bacteriocin production and immunity regions of pMRC01.

2.2. Sequence Homology of LtnJ

Analysis of the amino acid sequence of LtnJ reveals its homology to the zinc-containing alcohol dehydrogenase superfamily (Pfam00107). Specifically, LtnJ bears significant similarity to zinc-containing alcohol dehydrogenases and NAD(P)H dependent quinone oxidoreductases (COG0604). Amino acids 88-102 of LtnJ almost exactly match the signature sequence GHExxGxxxxx[G/A]xx[I/V/S/A/C] (domain 2) for alcohol dehydrogenases (Figure 16). As with quinone oxidoreductases, LtnJ contains a serine at position 89 in place of the conserved histidine of alcohol dehydrogenases. This histidine is required for binding of the zinc co-factor in zinc-dependent alcohol dehydrogenases. Furthermore, LtnJ lacks the cysteine residues present in alcohol dehydrogenases that are involved in binding of catalytic and structural zinc ions. Yet the amino acid sequence of LtnJ also does not completely match the NAD(P)H-binding signature sequence [G/S/D][D/E/Q/H]xxLxxx[S/A][S/A]GGxGxxxxQxx[K/R] (domain 4) for quinone oxidoreductases (Figure 16). Thus LtnJ represents a new class of reductases/dehydrogenases.

Interestingly, the sequences of other bacteriocin related proteins show homology with LtnJ. For example, SacJ, a protein associated with the production of the two-component lantibiotic staphylococcin C55 found in *Staphylococcus aureus*, is 47.8% identical to LtnJ.[120] Furthermore, PenN, a protein associated with the production of pediocin A produced by *Pediococcus pentosaceus*, is 40.4% identical to LtnJ.[121] The structures of staphylococcin C55 and pediocin A have not yet been elucidated and the function of SacJ and PenN have not been determined.

```
LtnJ        39-PKFD-9-VLVKINAISCNFRDKGIL-17-GSEFSGIVVDYGTNV-23-PGIPTN
SacD        38-PKFD-9-VLLQVKAISCNYRDKAIL-17-GSEFCASVISKGSNV-23--GIPTN
PenN        39-PKFD-9-VLVQVVAFSCNYRDKAIV-21-GSDFVGIIIEKGTNV-24-PGVVTN
Pfam00107   15-P----6-VLVKVLAAGICHSDLHLY-14-GHEGAGVVVEVGSGV-33-DGFGGF
COG0604     22-P----6-VLVRVKAAGVNPIDVLVR-14-GSEAAGVVVAVGSGV-17-DGG---
                  i          1  *           *      2            ii

LtnJ           FAS-14-PRELTDSEAACFPLGVQT-14-DPKPLIFSAKSATSLFII-47-VFD
SacD           FAS-14-PKNLTHNEAAGFSLGAQT-13-GGTPLVFSARSTTSIFLI-52-VFD
PenN           EAS-14-PDNMPNSIAAGYSIGGQT-12-SDKVLVLSARSNTSLFII-50-IFD
Pfam00107      TGD-17-PDGLPLEEAAALGCAGLT-12-GDTVLVHGA-GGVGLAAI-56-VLD
COG0604        YAE-12-PDGLSFEEAAALPLAGLT-13-GETVLVHGAAGGVGSAAI-54-VLD
                             3   *                4            iii

LtnJ           PFFD-15-YITCG-30-DISIIGNCLG-3-DLEE-38-GKC-10  (370)
SacD           PFFD-15-YITCG-30-NITIIGNCLG-3-DLED-37-GKC- 4  (365)
PenN           PFFD-15-YITCG-30-NISIIGNCIG-3-DLEN-37-GKV- 6  (371)
Pfam00107      CVGG-15-LVVVG-18-ERSIKGSFLG-5-ELRE-39-GKV- 4
COG0604        TVGG-15-LVSIG-18-RLTLRGVTLG-9-ALAE-37-GKV- 4
                       iv          v          vi     5
```

Figure 16. Alignment of LtnJ, SacJ, PenN, Pfam00107, and COG0604. Residues in red are 100% conserved and residues in blue are at least 50% conserved. Underlying numbers correspond to domains conserved in all cases, underlying Roman numerals correspond to domains conserved in LtnJ-like proteins, and underlying asterix are indicative of conserved His and Cys residues that coordinate the catalytic zinc atom in Zn-dependent alcohol dehydrogenases. The total number of residues are given in parentheses at the ends of the sequences (Reproduced from Cotter *et al.*).[117]

2.3. Introduction of LtnJ in *E. coli* Expression Systems

Sequence data of the plasmid pMRC01 from *L. lactis* subsp. *lactis* DPC3147 has made it possible to clone specific modification enzymes for high level expression in *E. coli* strains. Expression systems are designed to overproduce many copies of the desired protein within a host cell. The use of *E. coli* as a host cell imparts several advantages over *L. lactis*, such as ease of use, low cost, fast cell growth, and in many cases high protein expression. In the pET System[122], expression of the desired protein is controlled by a strong bacteriophage T7 transcription system using the T7*lac* promoter. The plasmid contains a T7 promotor and a *lac* operator sequence upstream of the protein gene sequence to be expressed. This plasmid is transformed into an expression host, such as *E. coli* BL21(DE3), containing a chromosomal copy of the T7 RNA polymerase gene. The expression of the T7 RNA polymerase gene is regulated by *lacUV5* promoter and is induced by addition of IPTG. IPTG binds to the *lac* repressor protein encoded by the *lacI* gene. This binding will inactivate the *lac* repressor protein and enable the transcription of the T7 RNA polymerase gene as well as the target gene (Figure 17). The T7 RNA polymerase is extremely selective and so active that almost all of the cells resources are converted to expression of the target gene. In some cases, the desired protein can equal to more than 50% of the total cell protein only a few hours after induction.[123]

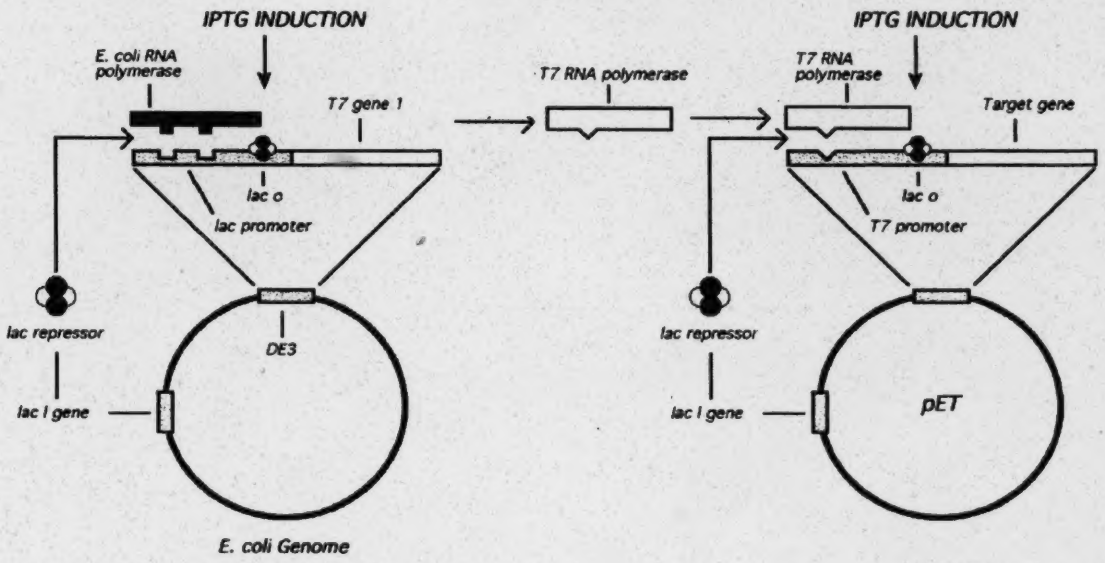


Figure 17. Control of the T7*lac* promoter in the pET System.

Dr. Paul Cotter (University College Cork, Ireland), created two such pET *E. coli* expression systems for LtnJ, one containing a N-terminal hexahistidine tag (pPC1) and one containing N- and C-terminal hexahistidine tags (pPC2). The presence of a hexahistidine tag allows for simplification of LtnJ purification as the protein can be separated from the other *E. coli* proteins using affinity chromatography. Both strains were generated using BL21(DE3) *E. coli* cells and the vector pET-32 Xa/LIC. In addition to hexahistidine, each system incorporated a long N-terminal linker followed by a Factor Xa cleavage site (Figure 18).

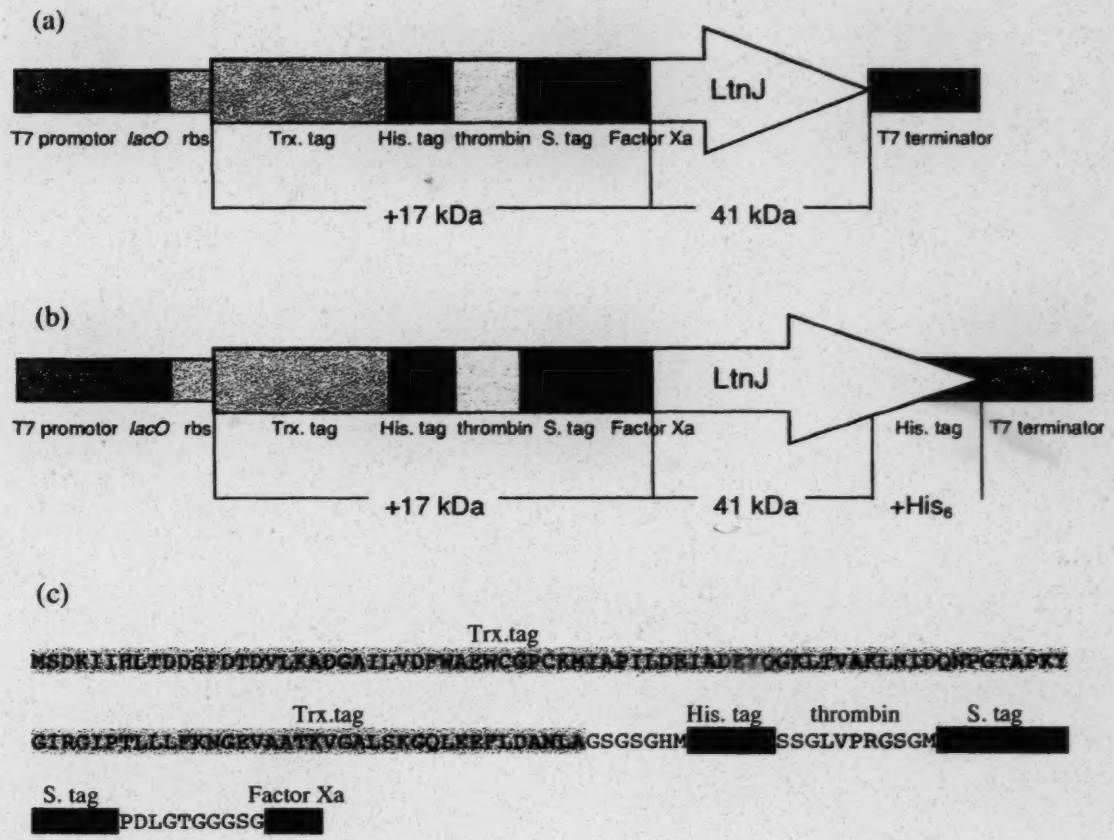


Figure 18. Coding region of (a) pPC1 and (b) pPC2 and (c) N-terminal tag amino acid sequence for pPC1 and pPC2.

2.4. Isolation of LtnJ from *E. coli* Containing pPC1 or pPC2

Initial attempts to express and purify LtnJ from BL21(DE3) *E. coli* cells containing pPC1 using standard growth conditions were unsuccessful as LtnJ is present in inclusion bodies when the cells are grown at 37 °C and 30 °C (*data not shown*). Inclusion bodies consist of improperly folded aggregated proteins. Although LtnJ can be purified from inclusion bodies, this requires denaturing conditions and subsequent refolding of the protein. Without a known activity assay for LtnJ, correct refolding cannot be monitored, thus production of soluble LtnJ is desirable. It is unknown why the overexpression of proteins can result in inclusion bodies. It is believed that they are formed when the bimolecular aggregation reaction of folding intermediates is faster than their rearrangement to the native conformation. Thus, if the speed of production of the recombinant protein is reduced, the folding intermediates may have more time to rearrange to their correctly folded conformation before finding another partially folded protein for aggregation. Lowering the growth temperature of the *E. coli* cells will reduce the speed of cell growth and subsequently protein synthesis, and perhaps decrease the formation of inclusion bodies. Therefore the BL21(DE3) *E. coli* cells containing pPC1 or pPC2 were grown at 15 °C, 20 °C, 25 °C, and 30 °C. The presence of soluble and insoluble LtnJ was monitored by Western Dot Blots using antibodies specific for the hexahistidine tags (Figure 19). To further confirm the presence of LtnJ, each fraction was analyzed by Western Blotting (Figure 20).

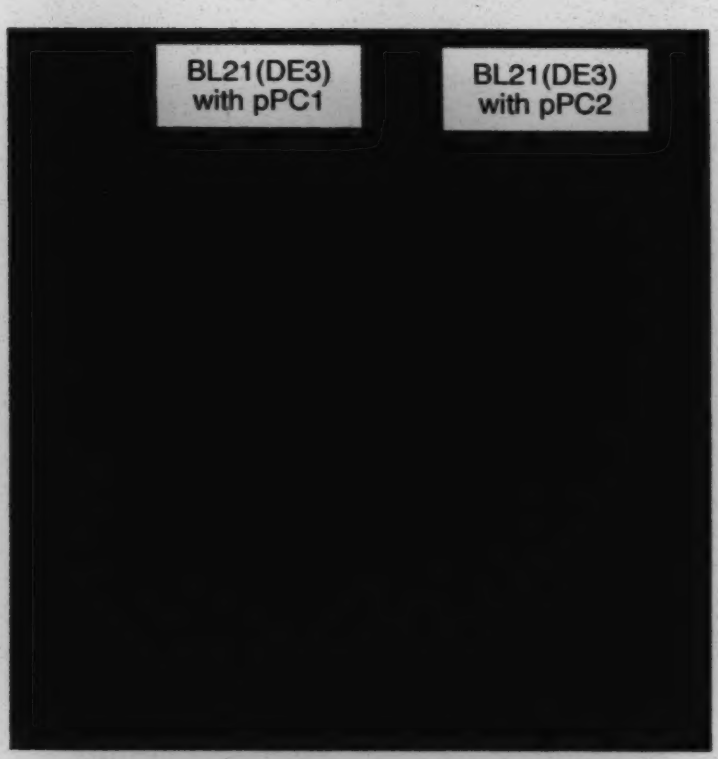


Figure 19. Western Dot Blots showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing either pPC1 or pPC2 grown at 15 °C, 20 °C, 25 °C, as well as at 30 °C for pPC2 (nothing was spotted for pPC1 at 30 °C).

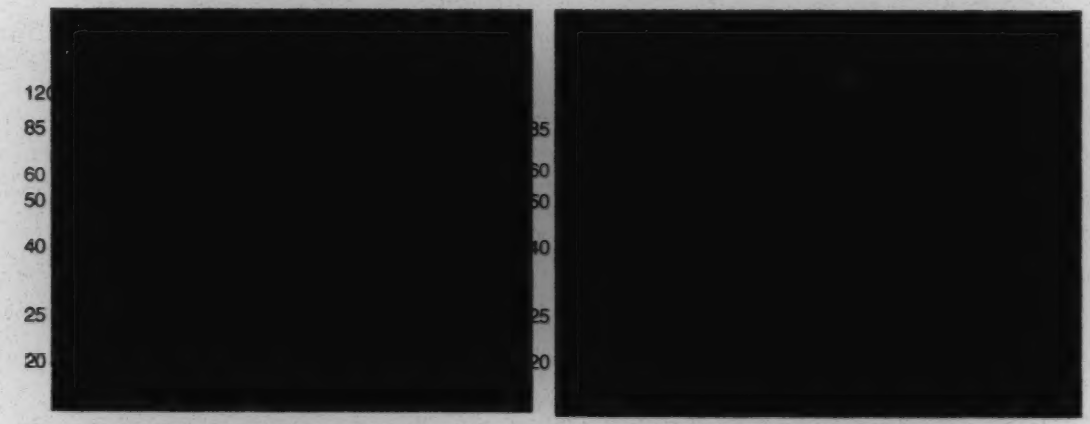


Figure 20. Western Blot analysis of the soluble and total protein fractions for *E. coli* BL21(DE3) containing either pPC1 (left) grown at 15 °C, 20 °C, and 25 °C or pPC2 (right) grown at 15 °C, 20 °C, 25 °C, and 30 °C. Lane 1; *molecular weight marker*, 2; *total protein at 15 °C*, 3; *soluble protein at 15 °C*, 4; *total protein at 20 °C*, 5; *soluble protein at 20 °C*, 6; *total protein at 25 °C*, 7; *soluble protein at 25 °C*, 8; *total protein at 30 °C*, 9; *soluble protein at 30 °C*.

Lowering the growth temperature results in some soluble LtnJ for both strains, although significant amounts of LtnJ are still present as insoluble protein. In a further attempt to improve LtnJ solubility, pPC1 and pPC2 were isolated from the *E. coli* BL21(DE3) cells and transformed into *E. coli* Origami™ B(DE3) cells. Origami™ B(DE3) cells contain mutations in the thioredoxin reductase and glutathione reductase genes, which together greatly enhance disulfide bond formation in the cytoplasm. This may help facilitate protein folding and result in a larger amount of soluble LtnJ. As with the BL21(DE3) strains, the two Origami™ B(DE3) transformants were grown at 15 °C, 25 °C, 30 °C, and 37 °C as well as at 17 °C for pPC1 and 20 °C for pPC2 transformants. The presence of soluble and insoluble LtnJ was monitored by Western Dot Blots (Figure 21). A Western Blot was also used to monitor LtnJ expression for Origami B(DE3) containing pPC1 (Figure 22).

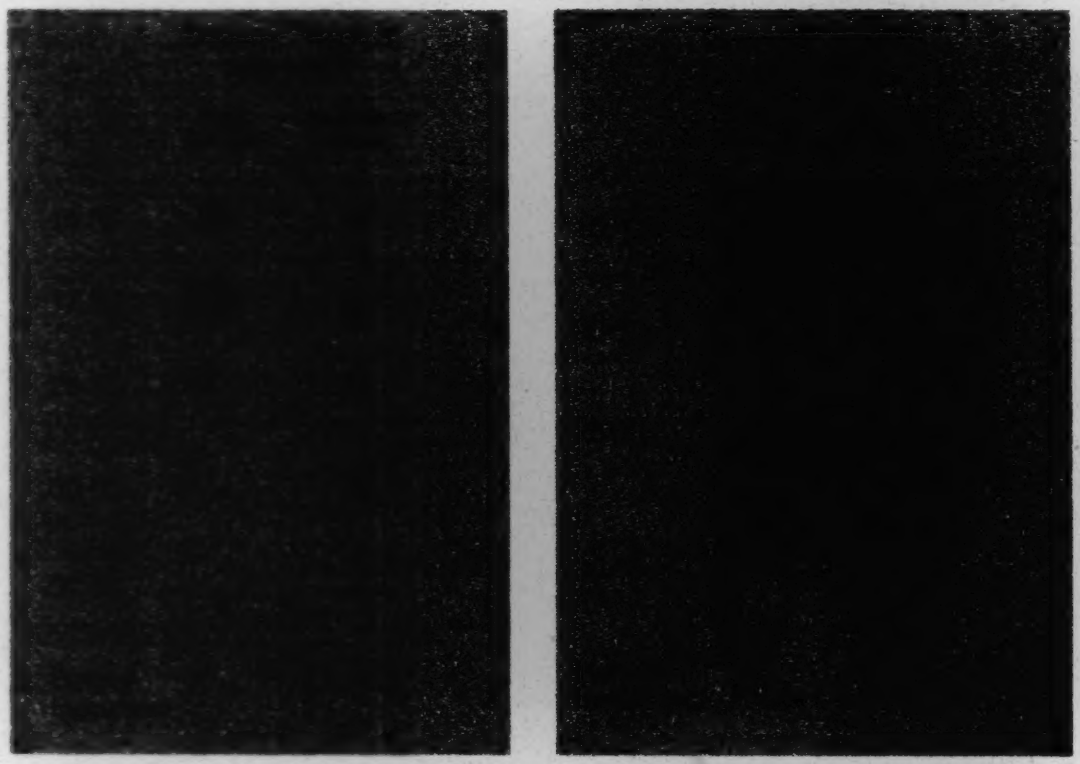


Figure 21. Western Dot Blots showing soluble and insoluble LtnJ production for *E. coli* Origami™ B(DE3) containing either pPC1 (right) or pPC2 (left) grown at various temperatures.

Figure 22. Western Blot analysis of the soluble and the insoluble fractions for *E. coli* Origami™ B(DE3) containing pPC1 grown at 15 °C, 17 °C, 25 °C, 30 °C, and 37 °C. Lane 1; *molecular weight marker*, 2; *total protein at 15 °C*, 3; *soluble protein at 15 °C*, 4; *total protein at 17 °C*, 5; *soluble protein at 17 °C*, 6; *total protein at 25 °C*, 7; *soluble protein at 25 °C*, 8; *total protein at 30 °C*, 9; *soluble protein at 30 °C*, 10; *soluble protein at 37 °C*.

Expression of LtnJ in Origami™ B(DE3) cells does not increase the amount of soluble protein. In fact, total LtnJ expression levels are diminished for both strains. Therefore purification via affinity chromatography of LtnJ was attempted from BL21(DE3) cells containing pPC1 grown at 20 °C. The sequence of purification steps is summarized in Figure 23.

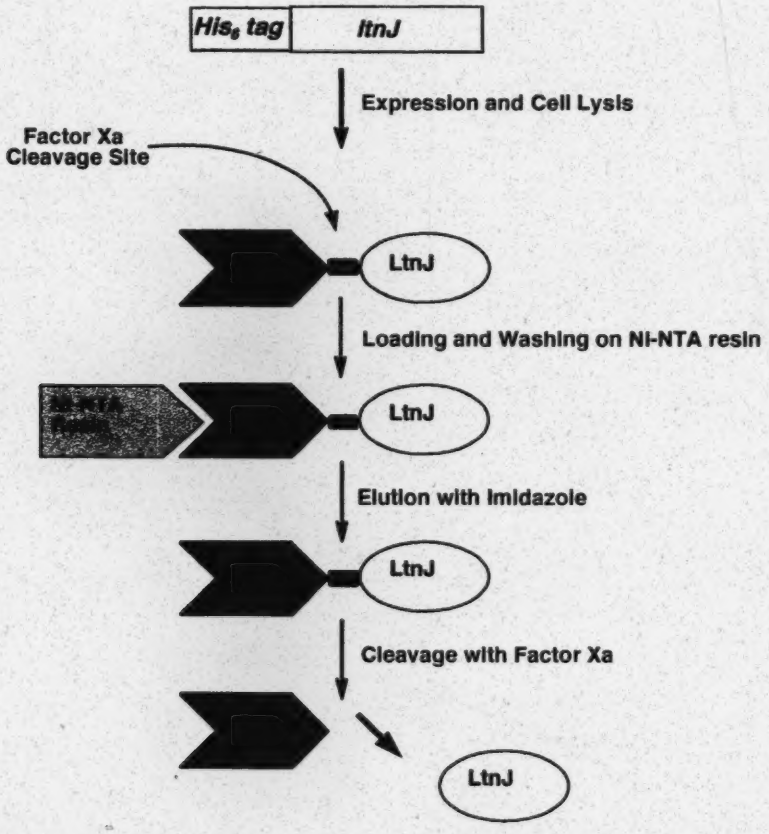


Figure 23. Affinity chromatography for the purification of LtnJ from *E. coli* BL21(DE3) cells containing pPC1.

Purification of LtnJ was moderately successful as analysis of the eluted fractions from the Ni-NTA column via SDS-PAGE indicates the presence of His_6-LtnJ as well as

proteins with a molecular weight smaller than LtnJ (Figure 24(a)). Upon closer analysis by Western Blotting, it is apparent that these additional protein bands are due to degraded LtnJ fragments still containing the hexahistidine tag (Figure 24(b)). Approximately 1 mg of fusion protein was obtained per one litre of cell culture.

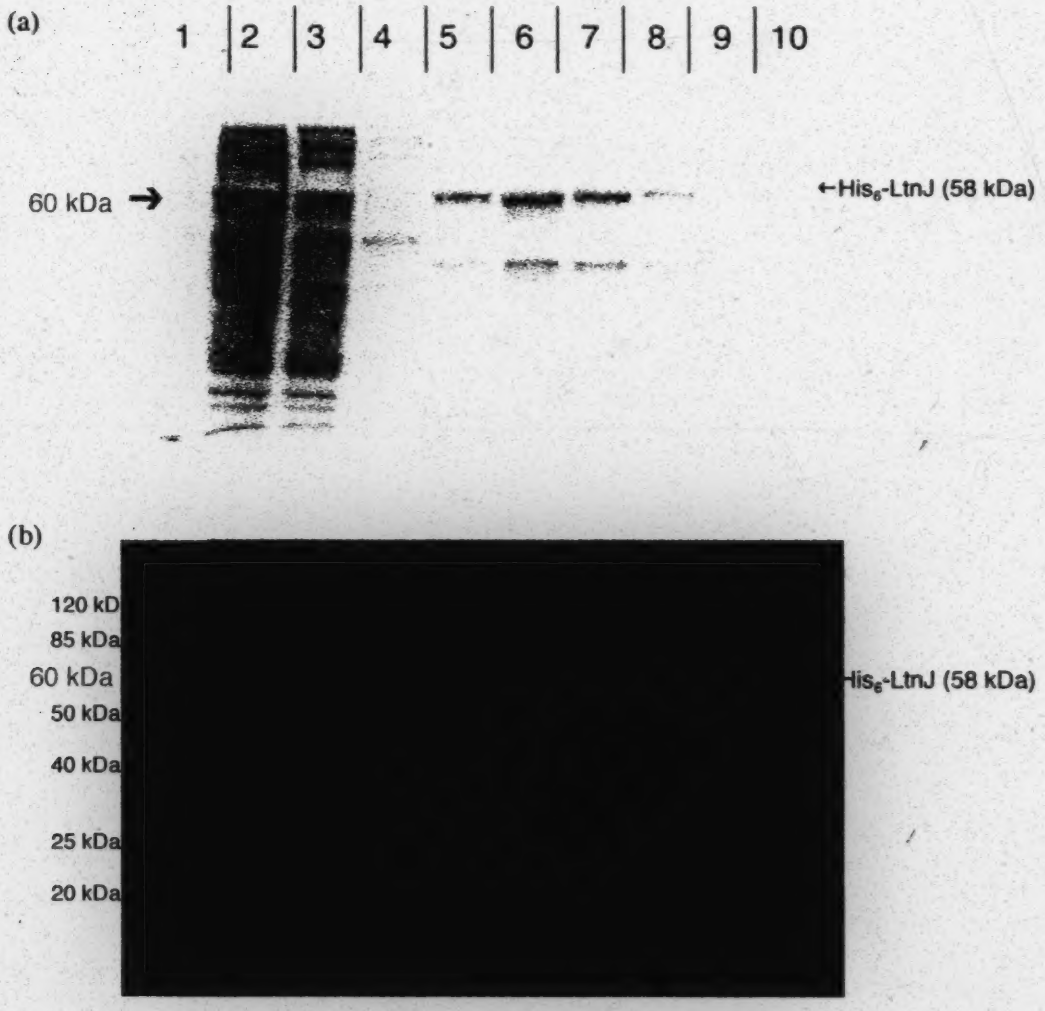


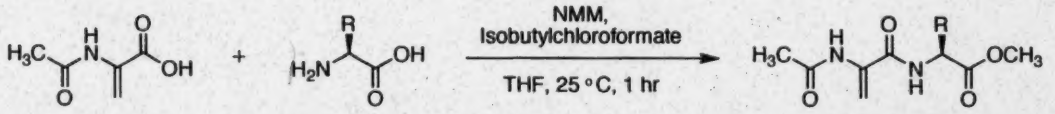
Figure 24. (a) SDS-PAGE and (b) Western Blot of fractions collected during the purification of LtnJ from *E. coli* BL21(DE3) cells containing pPC1. Lane 1; *molecular weight marker*, 2; *lysate*, 3; *Ni-NTA column flow-through*, 4; *wash fraction*, 5; *elution fraction 1*, 6; *elution fraction 2*, 7; *elution fraction 3*, 8; *elution fraction 4*, 9; *elution fraction 5*, 10; *elution fraction 6*.

2.5. Synthesis of Dipeptides

Examination of the primary sequence of LtnA1 and LtnA2 gives no insight into the substrate specificity of LtnJ as each D-alanine in lacticin 3147 is flanked on either side by a different amino acid. Even extending the analysis up to five residues on either side of each D-alanine provides no obvious residue homology. Furthermore, LtnA1 and LtnA2 lack dehydroalanine residues. Together, these facts suggest that LtnJ is a rather promiscuous enzyme and may not operate in a site-specific manner. However, it is worth noting that the recent *in vivo* analysis of LtnJ's substrate specificity has shown that it does not convert 2,3-dehydrobutyrines to D-aminobutyrate in place of 2,3-dehydroalanine residues.[117]

Dipeptides containing 2,3-dehydroalanine were chosen as the initial compounds to test as substrates of the purified LtnJ as they are the smallest peptides that can be synthesized. The dipeptides were modeled after the lacticin 3147 sequences; LtnA1 contains one D-alanine and LtnA2 contains two. Thus, the three dipeptides **24**, **25**, and **26** were synthesized by Dr. Steven Cobb (Vederas group, University of Alberta) via the coupling of the dehydroalanine containing compound **20** to the amino acids alanine (**21**), leucine (**22**), and isoleucine (**23**) (Scheme 3). In order to mimic a lacticin peptide, each dipeptide was synthesized such that it contains an acyl group on its N-terminus and a methyl ester on its C-terminus. In addition, D-alanine containing reference dipeptides **34**, **35**, and **36** were synthesized by Ian Armstrong, a summer student in the Vederas group (Scheme 4). The D-alanine containing dipeptides were synthesized via the coupling of

Boc-protected alanine (**27**) to methyl ester derivatives of alanine (**28**), leucine (**29**), and isoleucine (**30**) to give the Boc-protected dipeptides **31**, **32**, and **33**. Boc deprotection and incorporation of acyl groups on the N-termini yielded compounds **34**, **35**, and **36**.

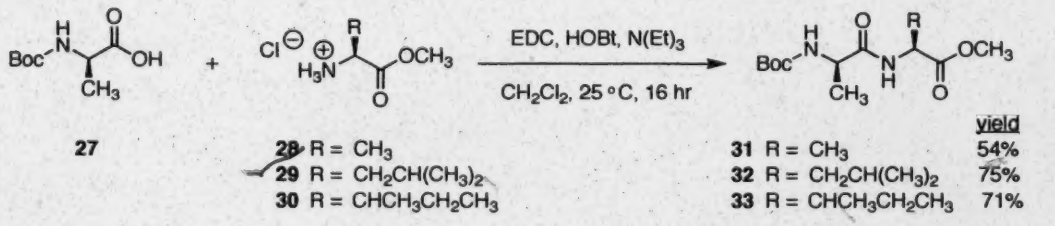


20

21 R = CH_3
22 R = $CH_2CH(CH_3)_2$
23 R = $CHCH_3CH_2CH_3$

	yield
24 R = CH_3	67%
25 R = $CH_2CH(CH_3)_2$	58%
26 R = $CHCH_3CH_2CH_3$	55%

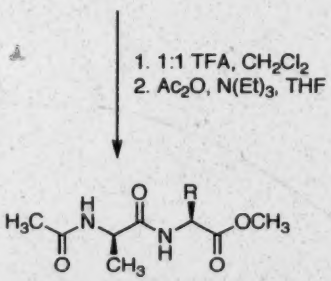
Scheme 3. Synthesis of 2,3-dehydroalanine containing dipeptides **24**, **25**, and **26**.



27

28 R = CH_3
29 R = $CH_2CH(CH_3)_2$
30 R = $CHCH_3CH_2CH_3$

	yield
31 R = CH_3	54%
32 R = $CH_2CH(CH_3)_2$	75%
33 R = $CHCH_3CH_2CH_3$	71%



	yield
34 R = CH_3	45%
35 R = $CH_2CH(CH_3)_2$	70%
36 R = $CHCH_3CH_2CH_3$	26%

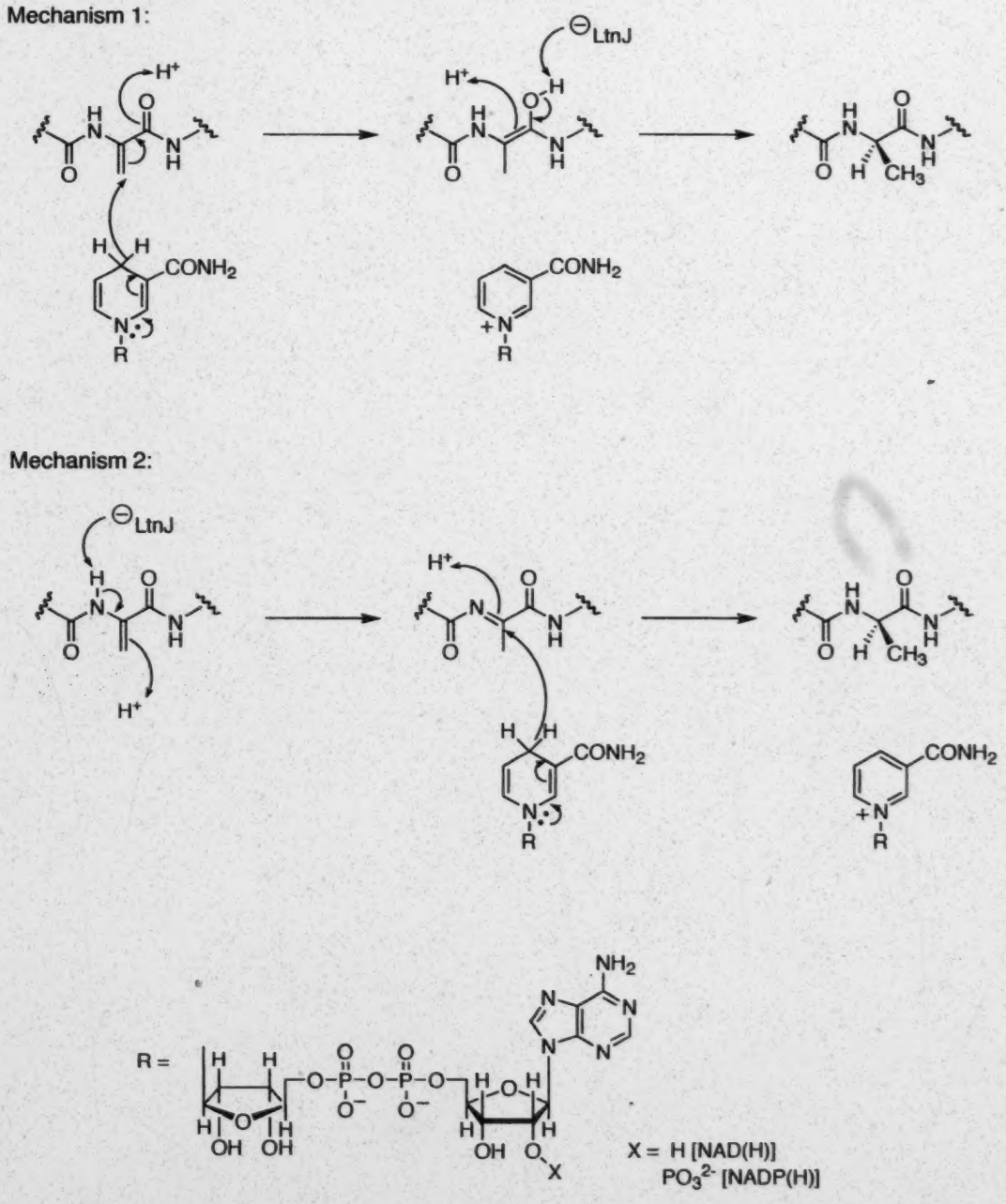
Scheme 4. Synthesis of D-alanine containing dipeptides **34**, **35**, and **36**.

2.6. Testing Dipeptides as Substrates of LtnJ from *E. coli* BL21(DE3) Containing pPC1

The conversion of 2,3-dehydroalanine to D-alanine requires the addition of two hydrogen atoms. Because LtnJ shows sequence homology with quinone oxidoreductases, the source of at least one of these hydrogen atoms may be from NADH or NADPH. Two possible mechanisms can be envisioned for this enzymatic transformation (Scheme 5). The first mechanism consists of Michael addition of a hydride from NAD(P)H onto the 2,3-dehydroalanine of the lacticin peptide to give an enol intermediate. A deprotonation/reprotonation sequence, possibly with the help of LtnJ, yields D-alanine. In the second mechanism, LtnJ assists in the formation of an imine which is converted to D-alanine via nucleophilic attack by a hydride from NAD(P)H.

With the synthesized dipeptides **24**, **25**, and **26** and LtnJ from BL21(DE3) containing pPC1 in hand, the reconstitution of enzymatic activity in vitro was attempted. The activity of many NAD(P)H-dependent enzymes is indirectly monitored using UV/Vis absorption spectroscopy since NAD(P)H absorbs 340 nm wavelength light whereas $NAD(P)^+$ does not.[124] Thus, initial attempts to measure the enzymatic activity of LtnJ were undertaken by incubating LtnJ (in either Tris or phosphate buffer, pH 5.5) with one of the dipeptides **24**, **25**, or **26** and either NADH or NADPH and monitoring the absorbance at 340 nm. The oxidation of NAD(P)H results in a decrease in absorbance at this wavelength. Control samples indicated that both NADH and NADPH were oxidized when incubated with LtnJ even in the absence of a dipeptide substrate showing that other

factors, besides LtnJ, contribute to the oxidation of NADH and NADPH. Hence an alternative method to monitor the activity of LtnJ was required.



Scheme 5. Possible mechanisms for the conversion of 2,3-dehydroalanine to D-alanine by LtnJ using NADH or NADPH as a co-factor.

Electrospray mass spectrometry (ESMS) is an important analytical tool for the detection of trace amounts of a compound.[125] Instead of using the indirect UV/Vis absorption assay to monitor LtnJ activity, a direct method using ESMS to detect the formation of D-alanine containing dipeptides **34**, **35**, and **36** was developed. In ESMS, the ionization of the analyte is accomplished by the formation of small, charged droplets from which positively charged ions on its surface are released into the gas phase upon evaporation of the solvent. If the concentration of salts is higher than the concentration of the analyte, the salts will be preferentially ionized, masking the analyte signal. Thus, in order to ensure ionization of the dipeptides, any salts or buffers necessary for the enzymatic reaction had to be removed prior to analysis. This was accomplished by lyophilizing the reactions and extracting the dipeptides with methanol. A series of reaction mixtures were prepared consisting of LtnJ (in either Tris or phosphate buffer, pH 5.5), one of the dipeptides **24**, **25**, or **26** as a substrate, and either NADH or NADPH as the proton source. An acidic pH of 5.5 was chosen for the assay conditions because LtnJ is found in a lactic acid producing organism. Each reaction was incubated at 30 °C, the optimal growth temperature for *L. lactis*, for 6 hours to allow sufficient time for the dehydroalanine containing dipeptides to be converted to their subsequent D-alanine containing enzyme products. Analysis of each reaction via ESMS revealed no product formation and only the presence of the dehydroalanine containing dipeptides **24**, **25**, and **26** (example spectra in Figure 25).

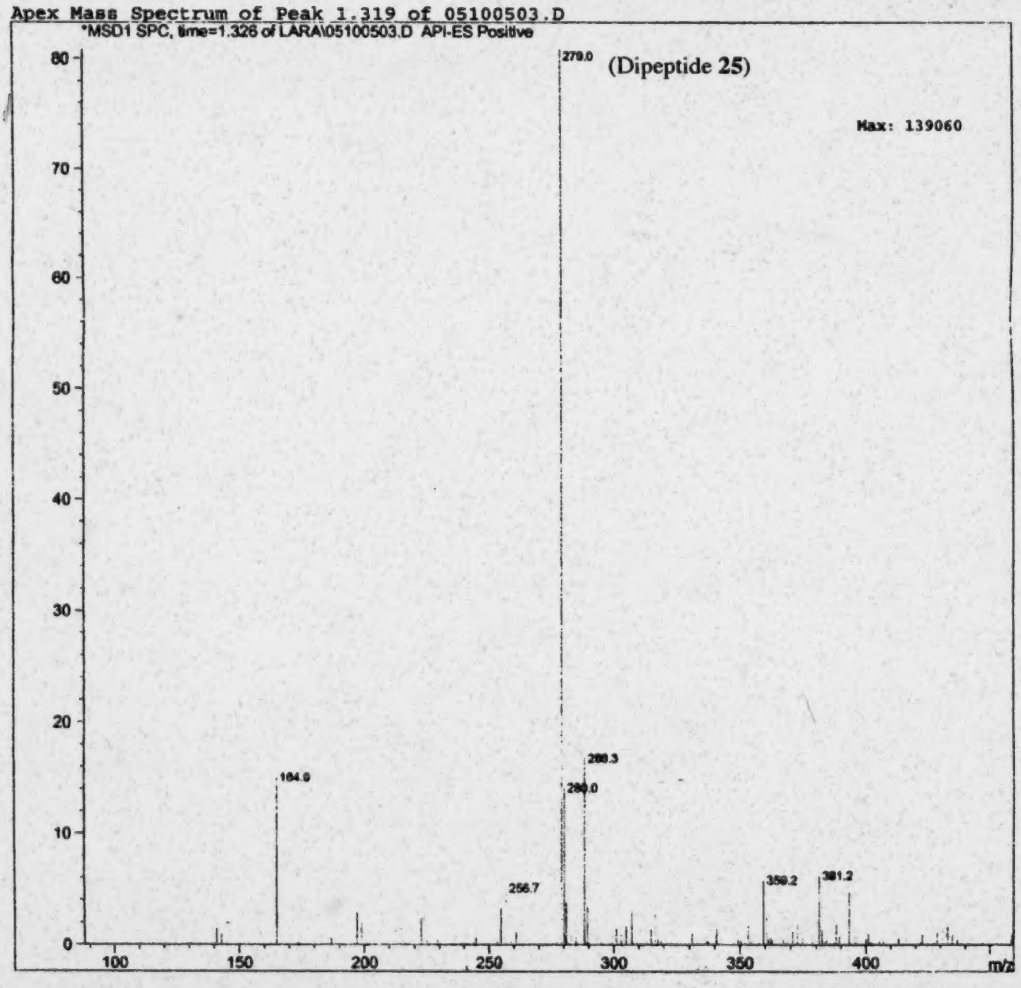


Figure 25. ESMS spectrum (80 eV) of a mixture containing 50 nmol dipeptide **25**, 100 nmol NADH, and 100 μg LtnJ from *E. coli* BL21 (DE3) containing pPC1 in a total volume of 200 μL phosphate buffer. Dipeptide **25** (m/z=279.0) is present and dipeptide **35** (m/z=281) is not detected.

There are a number of potential reasons why the formation of D-alanine containing dipeptides **34**, **35**, and **36** was not observed. Some of the possibilities include incorrect assay conditions (i.e. pH, type of buffer, temperature), the absence of a necessary co-factor or protein, inappropriate substrates, or inactive LtnJ due to incorrect

folding. Although any one, or combination, of these possibilities could be the reason for the lack of observed enzyme activity, inactive LtnJ seems probable. A closer study of the Western Blot of the lysate of the LtnJ producing *E. coli* cells (Figure 24(b)) reveals the presence of LtnJ fragments containing the hexahistidine tag. A fraction of the lysate was removed for SDS-PAGE and Western Blot analysis immediately after lysing the cells. The cell lysing buffer also contains a protease inhibitor cocktail to prevent rapid proteolysis of LtnJ by the *E. coli* proteases. This suggests that the formation of the LtnJ fragments may be occurring during protein synthesis, prior to cell lysis. The *E. coli* may be recognizing LtnJ as a foreign protein during, or shortly after, synthesis and thus proteolytically cleaving it inside the cell. Since there is no way to prevent this, the formation of new *E. coli* expression systems was undertaken.

2.7. Formation and Screening of New *E. coli* LtnJ Overexpression Strains

The recognition of foreign LtnJ by the *E. coli* may be due to the presence of a relatively long N-terminal linker (Figure 18). The total size of LtnJ is ~41 kDa while the N-terminal linker is ~17 kDa, yielding an expressed protein of ~58 kDa. To reduce the size of the linker, two pET *E. coli* expression systems were created, one with a N-terminal hexahistidine tag using pET-15b (pLS2) and one with a C-terminal hexahistidine tag using pET-11a (pLS1B). Both expression systems do not contain a cleavage site for removal of the hexahistidine tags, thus the size of the expressed protein is only slightly larger than LtnJ. A wide range of biologically active polyhistidine-tagged proteins has

been reported including lactate and galactose dehydrogenases.[126,127] These fusion enzymes are reported to be as functionally active as the wild-type enzymes. An additional pET *E. coli* expression system was created using pET-11a wherein LtnJ is left untagged (pLS1A). As mentioned in section 2.2, some pET expression systems are so successful that up to 50% of the total protein in the *E. coli* cells consists of the recombinant protein. In this situation, tagging the protein to simplify the purification procedure may, in fact, not be necessary. Therefore, this third pET expression system was created lest this be the case for LtnJ.

Simultaneously, a pMAL™ expression system[128,129] was constructed for LtnJ (pLS3). In this system, the recombinant protein contains a N-terminal maltose binding protein (MBP) followed by a Factor Xa cleavage site. Expression of the fusion protein is regulated by the p_{tac} promoter. The presence of a *lacI*^{q} gene, encoding for the *lac* repressor, keeps expression from p_{tac} low in the absence of IPTG. Although the MBP is a rather large protein, ~42 kDa, the pMAL™ expression system imparts several advantages over other expression systems. Typical yields are 10-40 mg per litre of cell culture, with up to 100 mg per litre observed for many proteins. A major advantage of the pMAL™ system is the enhanced solubility of fusion proteins expressed in *E. coli*.[130] Similar to hexahistidine-tagged proteins, purification of MBP-tagged proteins is achieved by affinity chromatography on an amylose column utilizing maltose as the eluent.

Each of the plasmids pLS1A, pLS1B, and pLS2 were transformed into *E. coli* BL21(DE3), chosen because it is deficient in proteases *lon* and *ompT*, and the plasmid

pLS3 was transformed into *E. coli* K12 TB1, chosen because of its compatibility with the pMAL™ expression system. The cells from each strain were grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. Strains containing plasmids pLS2, pLS1B, and pLS3 were monitored for the soluble production of LtnJ via Western Dot Blots and Western Blots using antibodies specific for either the hexahistidine tags or the MBP tag (Figures 26-28). Production of LtnJ by BL21(DE3) containing pLS1A was monitored by SDS-PAGE (Figure 29).

(a)

(b)

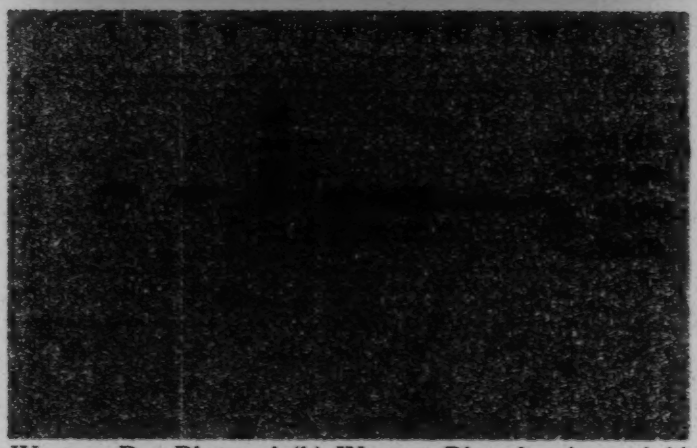


Figure 26. (a) Western Dot Blot and (b) Western Blot showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing pLS2 grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. Lane 1; *total protein 37 °C*, 2; *soluble protein at 37 °C*, 3; *total protein at 30 °C (4 h growth time)*, 4; *soluble protein at 30 °C (4 h growth time)*, 5; *total protein at 30 °C (17 h growth time)*, 6; *soluble protein at 30 °C (17 h growth time)*, 7; *total protein at 25 °C*, 8; *soluble protein at 25 °C*, 9; *total protein at 20 °C*, 10; *soluble protein at 20 °C*, 11; *total protein at 15 °C*, 12; *soluble protein at 15 °C*, 13; *molecular weight marker*.

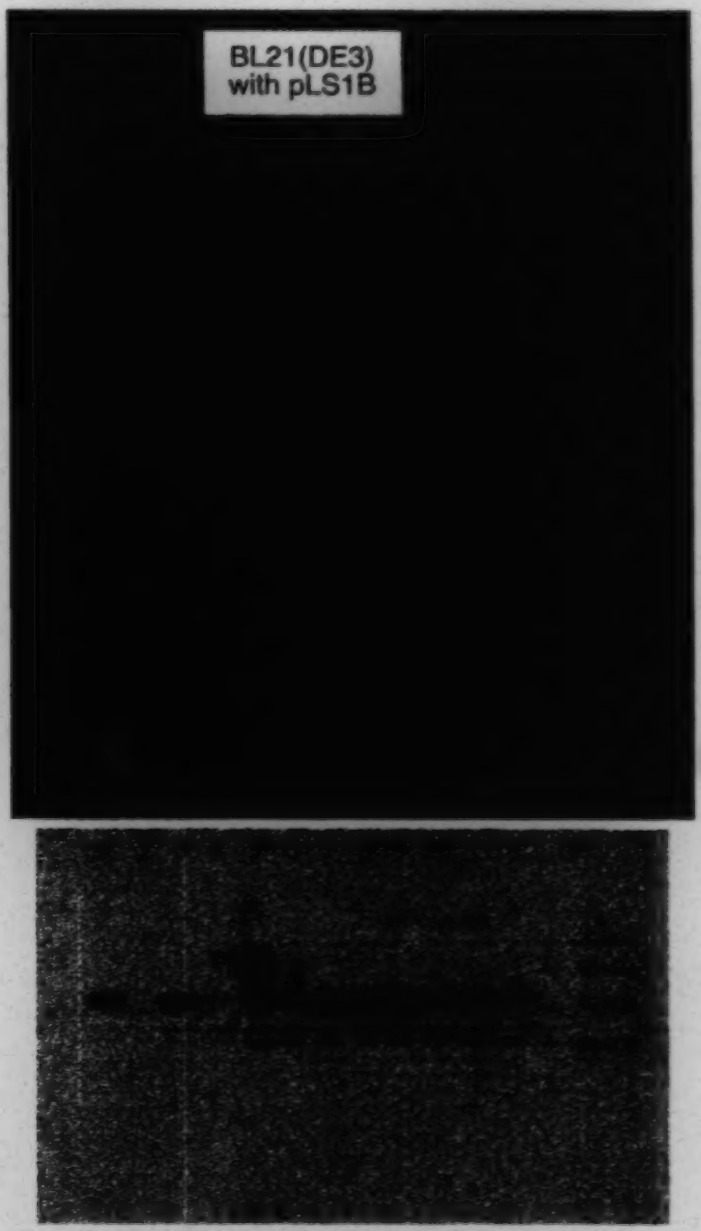


Figure 27. (a) Western Dot Blot and (b) Western Blot showing soluble and insoluble LtnJ production for *E. coli* BL21(DE3) containing pLS1B grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. Lane 1; *total protein at 37 °C*, 2; *soluble protein at 37 °C*, 3; *total protein at 30 °C (4 h growth time)*, 4; *soluble protein at 30 °C (4 h growth)*, 5; *total protein at 30 °C (17 h growth time)*, 6; *soluble protein at 30 °C (17 h growth time)*, 7; *total protein at 25 °C*, 8; *soluble protein at 25 °C*, 9; *total protein at 20 °C*, 10; *soluble protein at 20 °C*, 11; *total protein at 15 °C*, 12; *soluble protein at 15 °C*, 13; *molecular weight marker*.

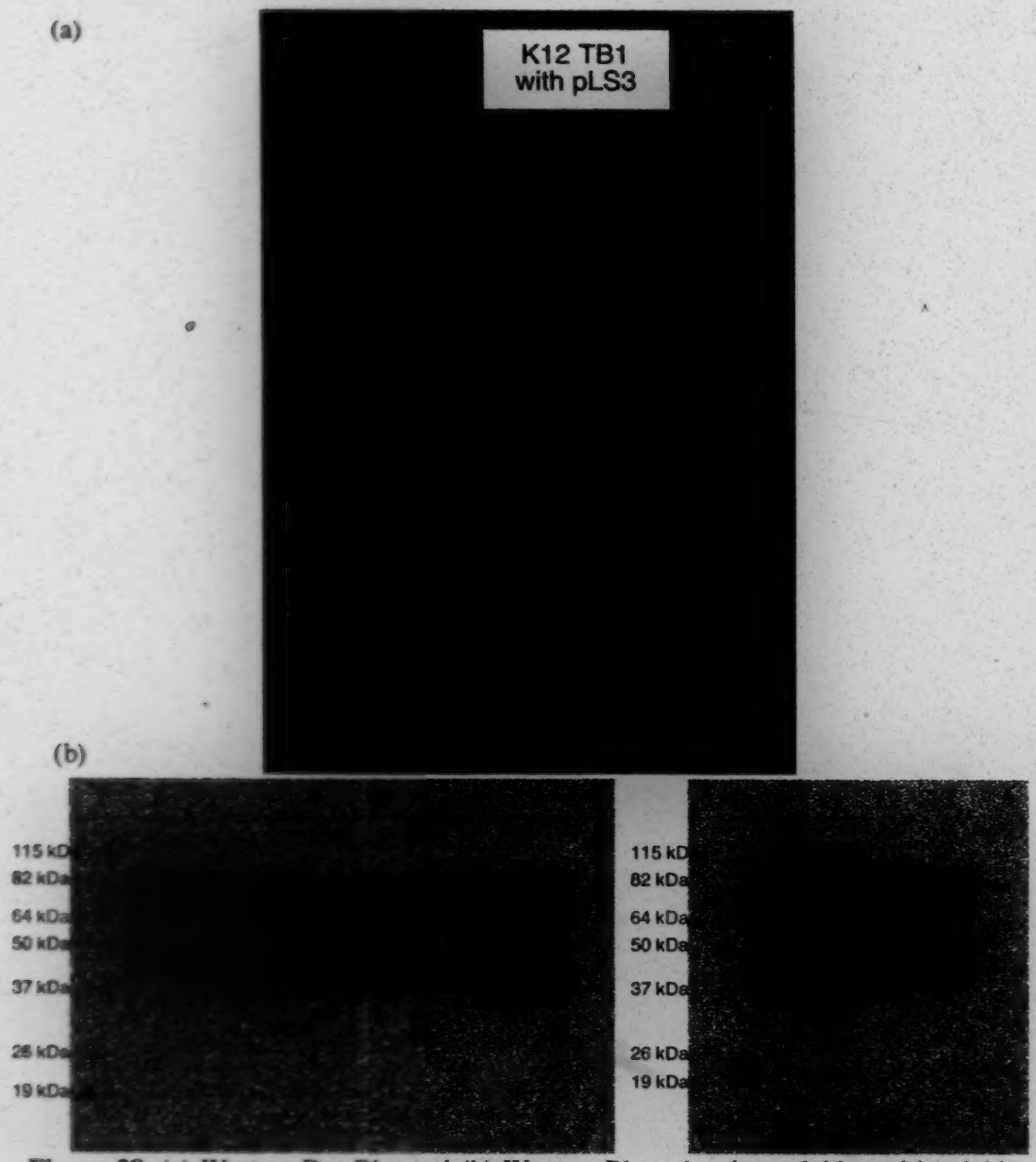


Figure 28. (a) Western Dot Blot and (b) Western Blots showing soluble and insoluble LtnJ production for *E. coli* K12 TB1 containing pLS3 grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. Lane 1; *molecular weight marker*, 2; *total protein at 37 °C*, 3; *soluble protein at 37 °C*, 4; *total protein at 30 °C (4 h growth time)*, 5; *soluble protein at 30 °C (4 h growth time)*, 6; *total protein at 30 °C (17 h growth time)*, 7; *soluble protein at 30 °C (17 h growth time)*, 8; *total protein at 25 °C*, 9; *soluble protein at 25 °C*, 10; *total protein at 20 °C*, 11; *soluble protein at 20 °C*, 12; *total protein at 15 °C*, 13; *soluble protein at 15 °C*.

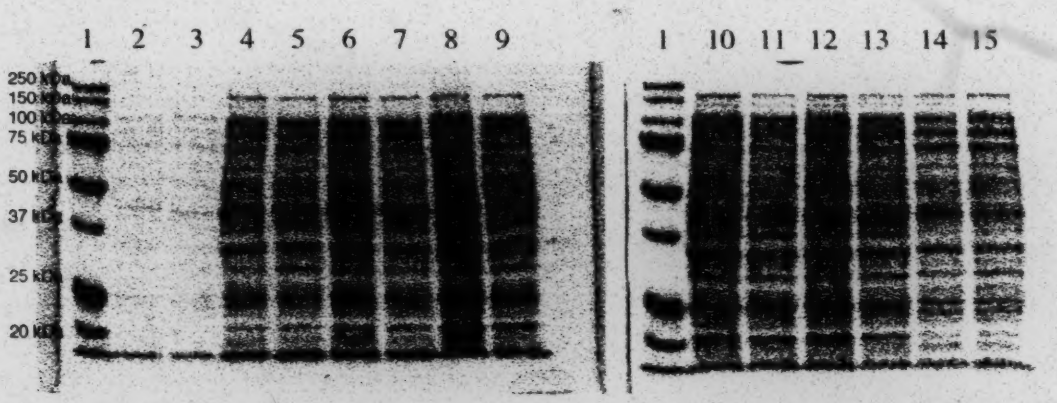


Figure 29. Coomassie-stained SDS-PAGE gels showing soluble and total protein fractions for *E. coli* BL21(DE3) containing pLS1A grown at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C. Lane 1; *molecular weight marker*, 2; *total protein before induction (control)*, 3; *soluble protein before induction (control)*, 4; *total protein* at *37 °C*, 5; *soluble protein at 37 °C*, 6; *total protein at 30 °C (4 h growth time)*, 7; *soluble protein at 30 °C (4 h growth time)*, 8; *total protein at 30 °C (17 h growth time)*, 9; *soluble protein at 30 °C (17 h growth time)*, 10; *total protein at 25 °C*, 11; *soluble protein at 25 °C*, 12; *total protein at 20 °C*, 13; *soluble protein at 20 °C*, 14; *total protein at 15 °C*, 15; *soluble protein at 15 °C*.

Western Blots and Dot Blots show that both hexahistidine-tagged strains only produce soluble LtnJ when the growth temperature is at or below 30 °C. In fact, cells grown at 30 °C do not show any soluble LtnJ unless they are grown for 17 hours. Even so, both strains yield low LtnJ expression levels. In contrast, the pMAL™ expression system provides soluble LtnJ at all growth temperatures with good expression yields. Although the Western Blot shows the presence of LtnJ degradation products, the amount of degradation varies amongst cell growth temperature with no clear correlation between amount of degradation and growth temperature. The untagged LtnJ expression system does not produce large enough amounts of LtnJ for simple purification from the *E. coli* proteins as it does not constitute a substantial portion of the total cell protein.

2.8. Optimization of Expression Strains for pLS3

As the pMAL™ expression system provides the most promising results for successful expression of recombinant LtnJ, the vector was screened for optimal expression strains. Altering the expression strain may decrease the amount of LtnJ degradation. In addition to K12 TB1, pLS3 was transformed into *E. coli* strains K12 CAG629, K12 UT5600, K12 ER2507, and K12 ER2508 by Dr. Sandra Marcus, Biochemistry Technologist, Department of Chemistry. The strains were chosen based on their compatibility with the pMAL™ expression system. Specifically, K12 CAG629 is deficient in the expression of heat shock proteins upon induction at 37 °C. This can increase the stability of foreign expressed proteins since a number of heat shock proteins affect proteolysis. K12 UT5600 is deficient in a periplasmic protease, OmpT, which may degrade proteins expressed in the periplasm or the cytoplasm upon cell lysis. Strain K12 ER2508 does not contain the major ATP-dependent protease found in the cytoplasm and can lead to increased stability of foreign expressed proteins.

Each strain was grown at 37 °C and screened for soluble and insoluble production of LtnJ by SDS-PAGE (Figure 30). In addition, LtnJ was purified from the soluble extract of each strain using amylose resin, following the protocol for pilot purification as described in the pMAL™ Protein Fusion and Purification manual (New England Biolabs Inc.), so that adequate comparisons of each strain could be made. The pilot purifications of strains K12 TB1, K12 UT5600, K12 ER2507, and K12 ER2508 all show reasonable expression levels of LtnJ and thus were further analyzed by Western Blots (Figure 31).

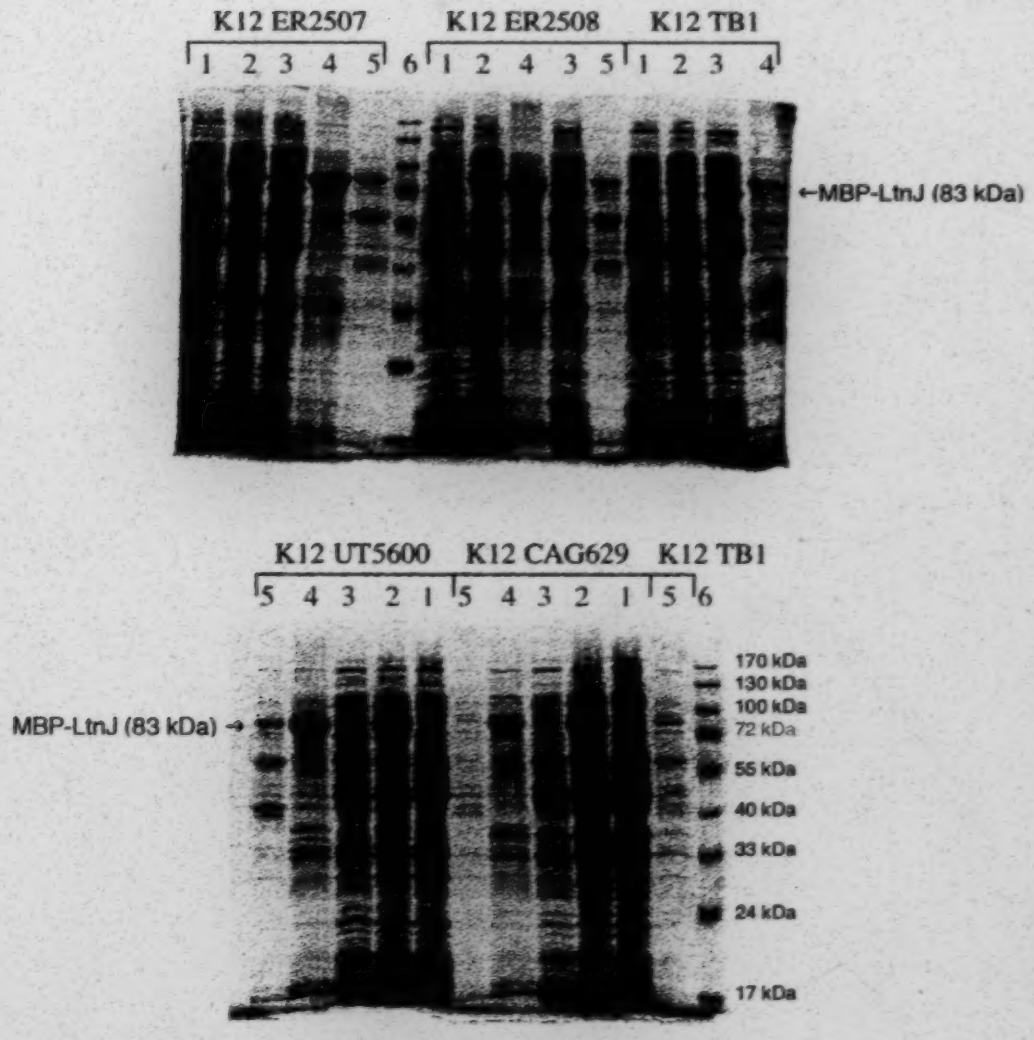


Figure 30. Coomassie-stained SDS-PAGE gels showing soluble, insoluble and total protein fractions and pilot purifications of LtnJ for *E. coli* K12 TB1, K12 CAG629, K12 UT5600, K12 ER2507, and K12 ER2508 containing pLS3 grown at 37 °C. Lane 1; *total protein before induction (control)*, 2; *total protein*, 3; *soluble protein*, 4; *insoluble protein*, 5; *pilot purification*, 6; *molecular weight marker*.

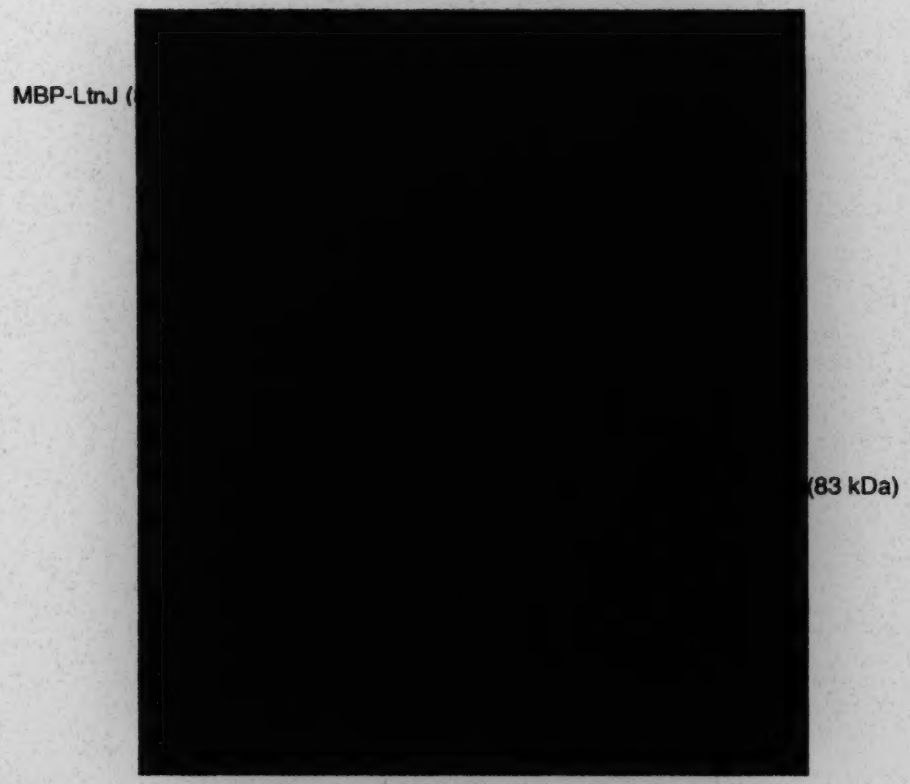


Figure 31. Western Blots showing soluble, insoluble and total protein fractions for *E. coli* K12 TB1, K12 UT5600, K12 ER2507, and K12 ER2508 as well as pilot purifications of LtnJ for *E. coli* K12 UT5600, K12 ER2507, and K12 ER2508 containing pLS3 grown at 37 °C. Lane 1; *total protein before induction (control)*, 2; *total protein*, 3; *soluble protein*, 4; *insoluble protein*, 5; *pilot purification*.

Both SDS-PAGE and Western Blots show that LtnJ expression levels and the amount of LtnJ degradation vary amongst strains. *E. coli* K12 CAG629 and K12 TB1 give the lowest amount of LtnJ expression with almost no soluble LtnJ expression for strain K12 CAG629. Strains K12 UT5600, K12 ER2507, and K12 ER2508 yield comparable LtnJ expression levels with most of the recombinant protein present in inclusion bodies. Examination of the Western Blots reveals minimal degradation of LtnJ

for all strains examined. Overall, strains K12 ER2507 and K12 ER2508 produce the highest amounts of soluble LtnJ, with the lowest amounts of degraded LtnJ, and yield the purest LtnJ from their pilot purification.

2.9. Purification of LtnJ from *E. coli* K12 ER2507 Containing pLS3

In light of the sequence homology of LtnJ with zinc-dependent dehydrogenases, it is possible that LtnJ is a metal-dependent enzyme. It has been demonstrated that some recombinant enzymes exhibit enhanced biological activity when expressed in growth media containing metal ions. For example, recombinant *E. coli* allantoinase, a metalloenzyme found in bacteria, fungi, and plants, as well as a few animals, displays the highest level of biological activity when extracted from cultures supplemented with zinc or cobalt.[131] Therefore, *E. coli* K12 ER2507 containing pLS3 was grown in Luria-Bertani media supplemented with zinc, iron, cobalt, nickel, copper, magnesium, manganese, molybdenum, and calcium to concentrations below those found to be toxic to the cells.[132]

Pilot LtnJ purifications were done on the soluble protein fractions from *E. coli* K12 ER2507 containing pLS3 grown at 25 °C and 30 °C in order to find the optimal growth temperature for soluble LtnJ expression. Analysis by SDS-PAGE reveals rather minor differences in LtnJ production between the two temperatures, with optimal conditions being at 30 °C (*data not shown*). Purification via affinity chromatography yielded approximately 10 mg of fusion protein per 1 litre of cell culture when the cells were grown at 30 °C. Optimal conditions for the cleavage of the MBP tag by Factor Xa

were determined by using 1%, 2%, or 3% (w/w ratio) Factor Xa (i.e. 1 mg Factor Xa for 100 mg fusion protein = 1%), incubating each reaction at 25 °C or 4 °C, and monitoring all reactions at 2, 4, 8, and 24 hour intervals by SDS-PAGE (Figures 32-34).

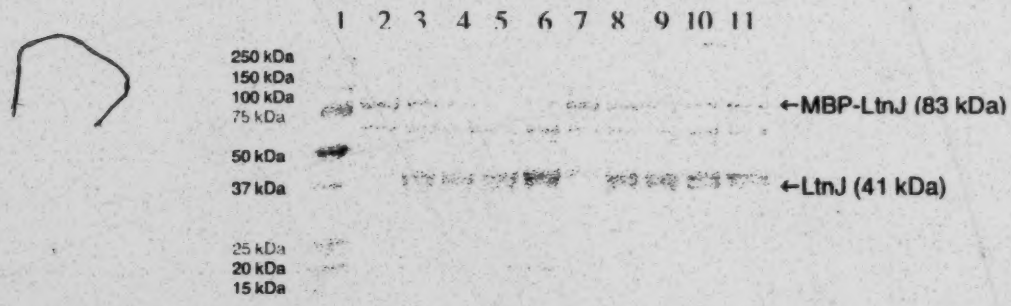


Figure 32. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 1% (w/w) Factor Xa incubated at 25 °C and 4 °C. Lane 1; *molecular weight marker*, 2; *25 °C, no Factor Xa (control)*, 3; *25 °C, 2 hrs*, 4; *25 °C, 4 hrs*, 5; *25 °C, 8 hrs*, 6; *25 °C, 24 hrs*, 7; *4 °C, no Factor Xa (control)*, 8; *4 °C, 2 hrs*, 9; *4 °C, 4 hrs*, 10; *4 °C, 8 hrs*, 11; *4 °C, 24 hrs*.

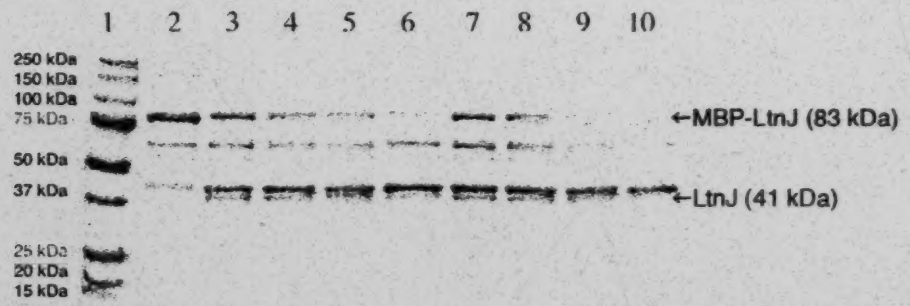


Figure 33. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 2% or 3% (w/w) Factor Xa incubated at 4 °C. Lane 1; *molecular weight marker*, 2; *no Factor Xa (control)*, 3; *2% Factor Xa, 2 hrs*, 4; *2% Factor Xa, 4 hrs*, 5; *2% Factor Xa, 8 hrs*, 6; *2% Factor Xa, 24 hrs*, 7; *3% Factor Xa, 2 hrs*, 8; *3% Factor Xa, 4 hrs*, 9; *3% Factor Xa, 8 hrs*, 10; *3% Factor Xa, 24 hrs*.

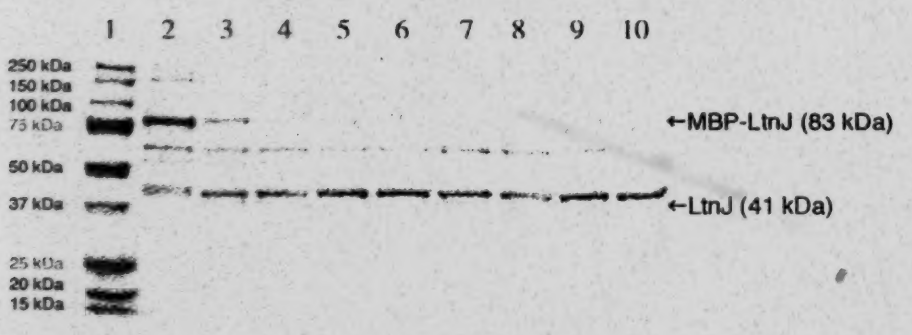


Figure 34. Coomassie-stained SDS-PAGE gel of pilot MBP cleavage reactions using 2% or 3% (w/w) Factor Xa incubated at 25 °C. Lane 1; *molecular weight marker*, 2; *no Factor Xa (control)*, 3; *2% Factor Xa, 2 hrs*, 4; *2% Factor Xa, 4 hrs*, 5; *2% Factor Xa, 8 hrs*, 6; *2% Factor Xa, 24 hrs*, 7; *3% Factor Xa, 2 hrs*, 8; *3% Factor Xa, 4 hrs*, 9; *3% Factor Xa, 8 hrs*, 10; *3% Factor Xa, 24 hrs.*

The cleavage of LtnJ can be monitored by observing the disappearance of the fusion protein at ~83 kDa. Cleavage occurs more quickly and more completely at 3% (w/w) Factor Xa. Although LtnJ does not contain the Factor Xa recognition sequence (i.e. I[E/D]GR), analysis of each cleavage reaction shows some loss of LtnJ (~41 kDa) after 24 hours when incubated at 4 °C and after 4 hours when incubated at 25 °C. This loss is most likely due to non-specific cleavage of LtnJ by Factor Xa and is most pronounced with longer incubation times, higher temperatures, and higher concentrations of Factor Xa. This protease is known to cleave after basic residues other than the arginine found in its recognition sequence, depending on the conformation of the protein substrate.[133,134] Thus optimal cleavage of MBP from the LtnJ-fusion occurs at a fine balance between complete MBP cleavage and non-specific LtnJ cleavage. The most favorable incubation conditions were determined to be 6 hours at 4 °C using 2% (w/w) Factor Xa. Under these conditions, the final reaction mixture contains free LtnJ as well

as some MBP-fused LtnJ. Immediately following the cleavage reaction, Factor Xa is removed with Xa removal resin in order to prevent further cleavage of LtnJ. Any residual Factor Xa is then irreversibly inhibited with the serine protease inhibitor AEBSF.

2.10. Testing Dipeptides as Substrates of LtnJ from *E. coli* K12 ER2507 Containing pLS3

LtnJ partially purified from *E. coli* K12 ER2507 containing pLS3 was then tested for biological activity with the synthesized dipeptides **24**, **25**, and **26**. Once again, a series of reactions were prepared consisting of LtnJ (in either Tris or phosphate buffer, pH 5.5), one of the dipeptides **24**, **25**, or **26** as a substrate, either NADH or NADPH as the hydride source, and either none or one of the following co-factors; zinc, magnesium, iron, FAD and iron, EDTA, and DTT. As before, the reactions were incubated at 30 °C for 6 hours, lyophilized, and the dipeptides were extracted with methanol. Analysis by ESMS once again results in the presence of only the dehydroalanine containing dipeptides **24**, **25**, and **26**. The formation of D-alanine containing dipeptides **34**, **35**, and **36** is not observed (example spectra in Figures 35 and 36).

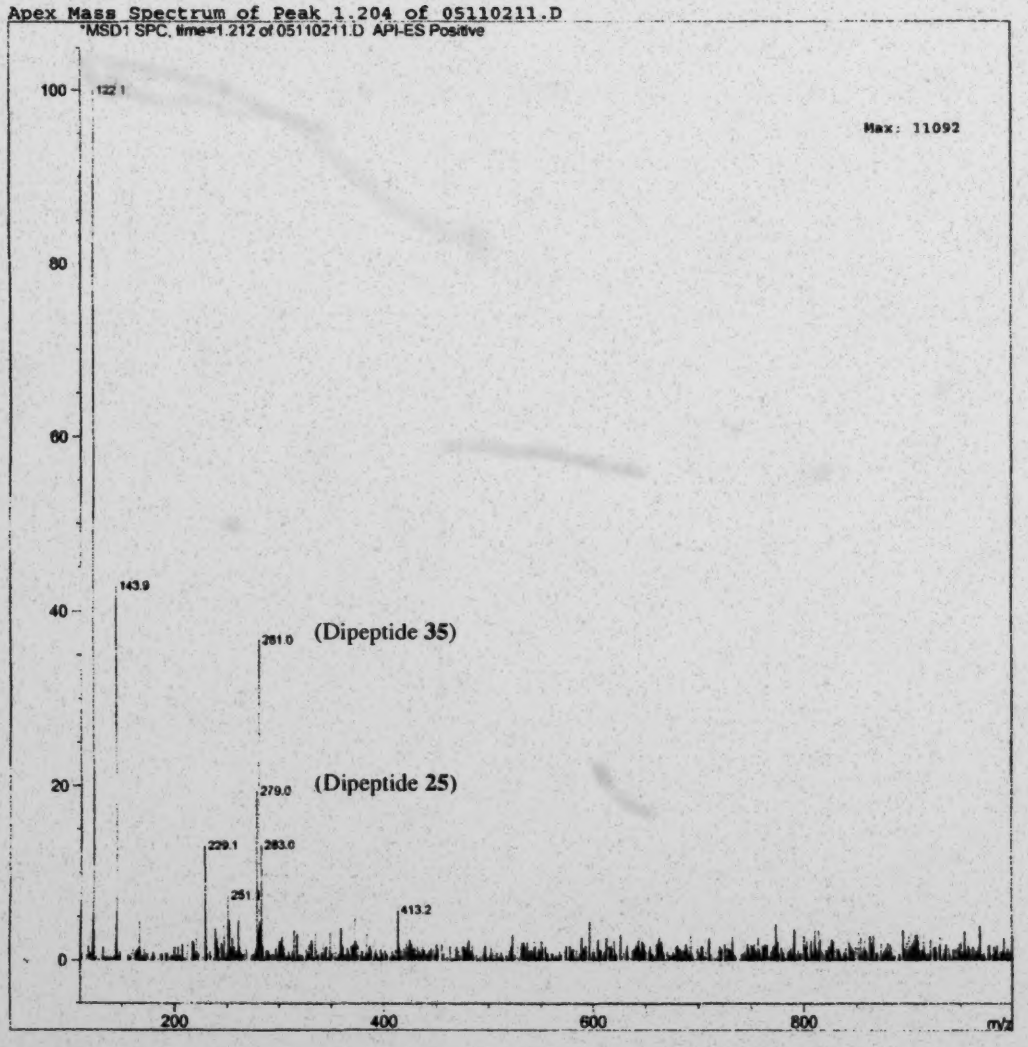


Figure 35. ESMS spectrum (80 eV) of control mixture containing 50 nmol dipeptide **25** with 50 nmol dipeptide **35** added, 100 nmol NADH, 100 nmol NADPH, and 100 μg LtnJ from *E. coli* ER2507 containing pLS3 in a total volume of 200 μL Tris buffer. Both dipeptides **25** (m/z=279) and **35** (m/z=281) are detectable under these conditions.

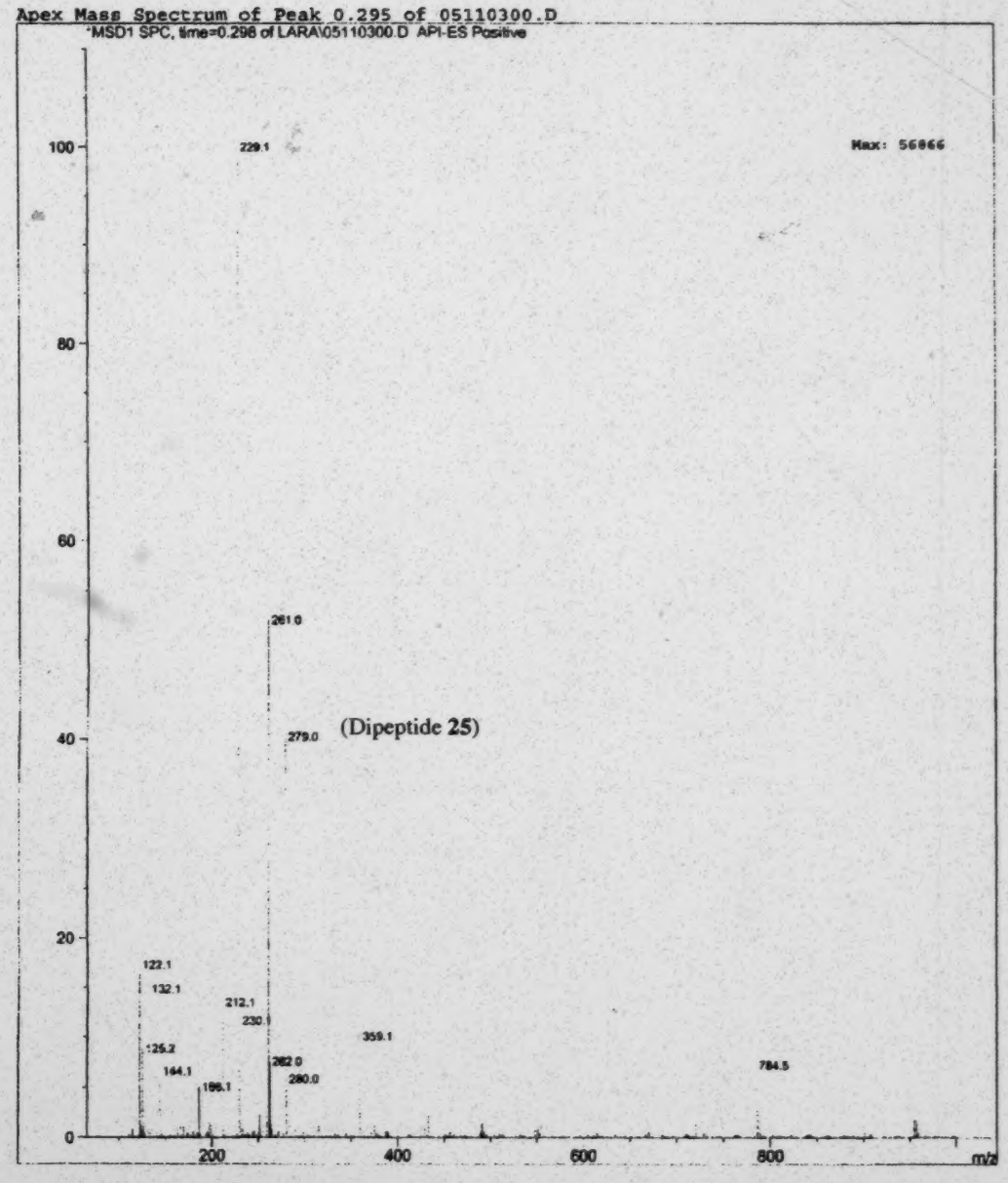


Figure 36. ESMS spectrum (80 eV) of a mixture containing 50 nmol dipeptide **25**, 100 nmol NADH, 100 nmol NADPH, and 100 μg LtnJ from *E. coli* ER2507 containing pLS3 in a total volume of 200 μL Tris buffer. Dipeptide **25** (m/z=279) is present and the desired dipeptide product **35** (m/z=281) is not detected.

2.11. LtnJ from the Cell Free Extract of *L. lactis*

Thus far, two overexpression systems have been successful in the isolation of recombinant LtnJ. However, the *in vitro* reconstitution of biological activity of LtnJ from each of these systems has been unsuccessful. Consequently, LtnJ from its natural producing strain, *L. lactis* subsp. *lactis* MG1363, was investigated for its biological activity with the synthesized dipeptides. If LtnJ from *L. lactis* is biologically active using the same assay conditions previously tested for LtnJ from the overexpression systems, then the recombinant proteins are inactive. On the other hand, if LtnJ from *L. lactis* does not show activity, the lack of observed activity from the recombinant proteins may be caused by other factors.

Unlike the *E. coli* overexpression strains, purification of LtnJ from *L. lactis* is difficult because its expression levels are not enhanced. As a result, a crude mixture of LtnJ can be prepared by the cell lysis of *L. lactis* subsp. *lactis* MG1363 and subsequent collection of the supernatant to give the cell free extract (CFE). This extract contains all the proteins present in *L. lactis* and thus should also contain LtnJ, although without a viable activity assay its presence cannot be confirmed. A cocktail of protease inhibitors is added to the cell lysis buffer prior to lysing to prevent the degradation of LtnJ by *L. lactis* proteases. The CFE yields approximately 25 mg of protein per one litre of cell culture at a concentration of ~0.5 mg/mL.

2.12. Testing Dipeptides as Substrates of LtnJ from CFE of *L. lactis*

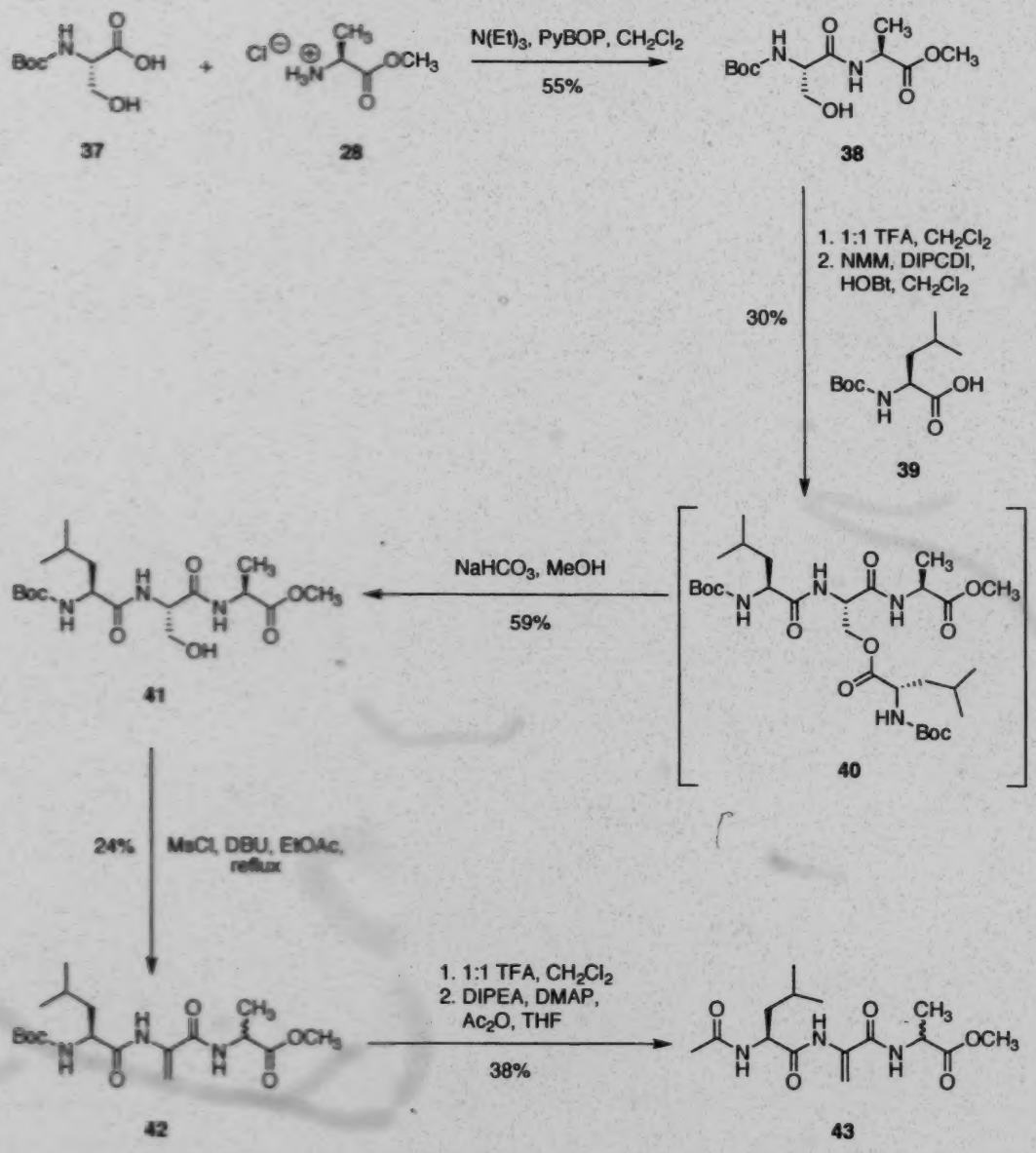
The same series of reactions were prepared for LtnJ from *L. lactis* as for LtnJ from *E. coli* K12 ER2507. A total of 4 mL of CFE in Tris buffer, pH 5.5 was used in the assay, which is equivalent to ~2 mg of total protein. Analysis by ESMS yet again reveals the absence of D-alanine containing dipeptides **34**, **35**, and **36** (*data not shown*).

2.13. Synthesis of Tripeptides

It may be that the dipepitdes **24**, **25**, and **26** are simply too small for recognition by LtnJ. Also, each dipeptide was prepared in such a way that its N-terminus is a dehydroalanine. Thus the synthesis of three dehydroalanine containing tripeptides **43**, **53**, and **54** was examined, once again modeling the primary sequences of LtnA1 and LtnA2 to create three new potential LtnJ substrates (Schemes 6 and 7).

The tripeptide **43** was prepared by the coupling of Boc-protected serine (**37**) to a methyl ester derivative of alanine (**28**) to give the dipeptide **38** in moderate yield (Scheme 6). Boc deprotection of compound **38** followed by coupling to Boc-protected leucine (**39**) lead to the undesired formation of a di-coupled leucinyl compound **40**, which was converted to the desired mono-coupled tripeptide **41** via ester hydrolysis. Subsequent mesylation and treatment with base yielded the Boc-protected dehydroalanine containing tripeptide **42**.[135] The unforeseeable formation of a diastereomeric mixture of coupound **42** was detected via ^{1}H NMR. Although it is not known which center has isomerized, the

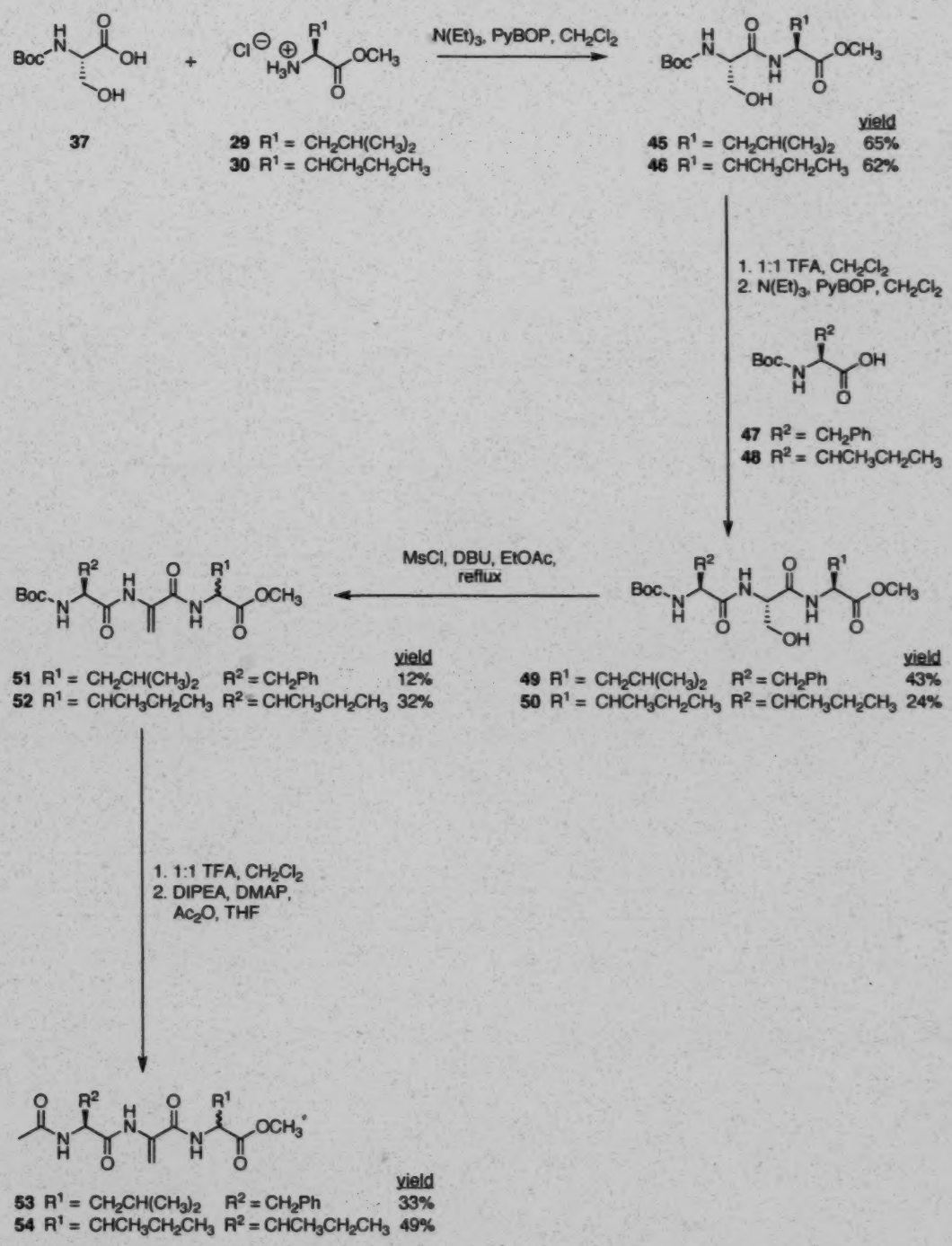
most acidic chiral proton in compound **41** is the alpha proton of the alanine residue. The formation of diastereomers may be the consequence of base abstraction of this alpha proton followed by re-protonation, resulting in a loss of chirality at this center. Attempts to separate the stereoisomers using RP-HPLC were unsuccessful, thus the mixture was reacted further through the last step in the synthesis of the tripeptide in hopes of creating a separable mixture. The final step consisted of Boc deprotection followed by treatment with acetic anhydride in basic conditions to afford a diastereomeric mixture of the N-acylated tripeptide **43**. Once again, attempts to separate the diastereomers of compound **43** using RP-HPLC were unsuccessful. As the desired isomer (i.e. consisting of L-amino acids) is present in the diastereomeric mixture of compound **43**, this mixture was used in the development of an LtnJ assay.



37 28 38 39 40 41 42 43

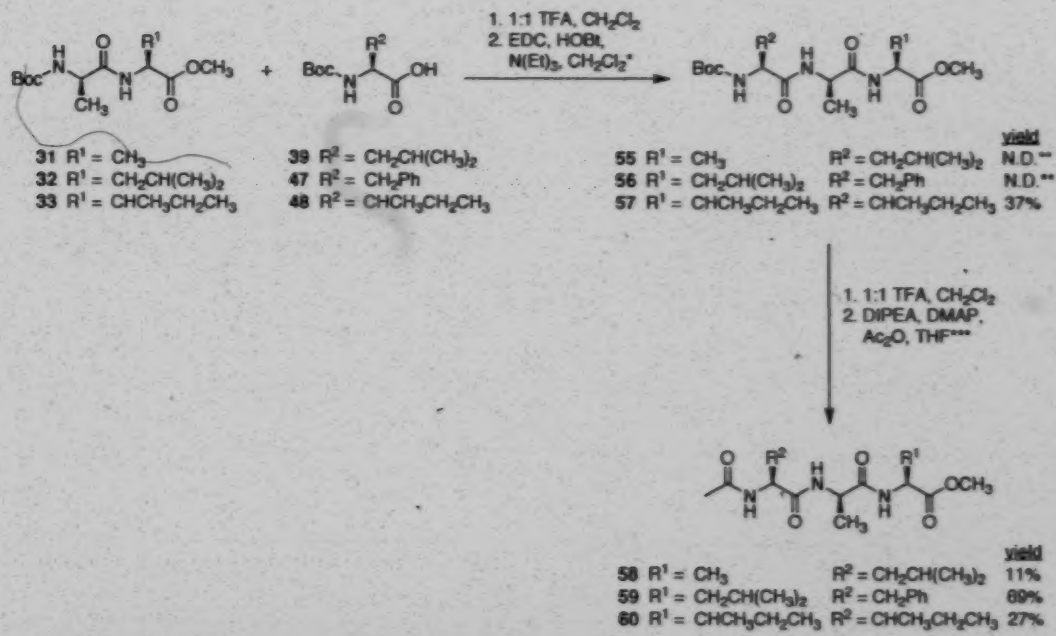
Scheme 6. Synthesis of 2,3-dehydroalanine containing tripeptide **43**.

The tripeptides **53** and **54** were generated in a similar manner as compound **43** starting with the coupling of Boc-protected serine (**37**) to the methyl ester of either leucine (**29**) or isoleucine (**30**) to give the dipeptides **45** and **46**, respectively (Scheme 7). Boc deprotection of **45** and **46** followed by coupling to either Boc-protected phenylalanine (**47**) or Boc-protected isoleucine (**48**) gave the tripeptides **49** and **50**, respectively, in low yields. The low yields may be the result of undesirable di-coupling of the Boc-protected phenylalanine (**47**) and isoleucine (**48**) to the hydroxy group of the serine residue, as was observed en route to the synthesis of compound **41** (Scheme 6). The tripeptides **49** and **50** were then mesylated and treated with DBU to yield the dehydroalanine containing tripeptides **51** and **52**.[135] As in the synthesis of compound **42**, treatment of compounds **49** and **50** with base resulted in the isomerization of a chiral centre as determined by ^{1}H and ^{13}C NMR analysis of compounds **51** and **52**. Once again, these diastereomeric mixtures of compounds **51** and **52** were not separable by the RP-HPLC methods employed and were Boc deprotected and acylated to yield the diastereomeric compounds **53** and **54**. Attempts to separate these stereoisomers using RP-HPLC were also unsuccessful and thus the mixtures were used in the development of an LtnJ assay.


N(Et)3, PyBOP, CH2Cl2
37
29 R1 = CH2CH(CH3)2
30 R1 = CHCH3CH2CH3
yield
45 R1 = CH2CH(CH3)2 65%
46 R1 = CHCH3CH2CH3 62%
1. 1:1 TFA, CH2Cl2
2. N(Et)3, PyBOP, CH2Cl2
47 R2 = CH2Ph
48 R2 = CHCH3CH2CH3
MsCl, DBU, EtOAc,
reflux
yield
49 R1 = CH2CH(CH3)2 R2 = CH2Ph 43%
50 R1 = CHCH3CH2CH3 R2 = CHCH3CH2CH3 24%
yield
51 R1 = CH2CH(CH3)2 R2 = CH2Ph 12%
52 R1 = CHCH3CH2CH3 R2 = CHCH3CH2CH3 32%
1. 1:1 TFA, CH2Cl2
2. DIPEA, DMAP,
Ac2O, THF
yield
53 R1 = CH2CH(CH3)2 R2 = CH2Ph 33%
54 R1 = CHCH3CH2CH3 R2 = CHCH3CH2CH3 49%

Scheme 7. Synthesis of 2,3-dehydroalanine containing tripeptides **53** and **54**.

The D-alanine containing reference tripeptides **58**, **59**, and **60** were generated in a similar manner as the dehydroalanine containing tripeptides (Scheme 8).


1. 1:1 TFA, CH_2Cl_2
2. EDC, HOBt,
$N(Et)_3$, CH_2Cl_2*
31 $R^1 = CH_3$
32 $R^1 = CH_2CH(CH_3)_2$
33 $R^1 = CHCH_3CH_2CH_3$
39 $R^2 = CH_2CH(CH_3)_2$
47 $R^2 = CH_2Ph$
48 $R^2 = CHCH_3CH_2CH_3$
yield
55 $R^1 = CH_3$ $R^2 = CH_2CH(CH_3)_2$ N.D.**
56 $R^1 = CH_2CH(CH_3)_2$ $R^2 = CH_2Ph$ N.D.**
57 $R^1 = CHCH_3CH_2CH_3$ $R^2 = CHCH_3CH_2CH_3$ 37%
1. 1:1 TFA, CH_2Cl_2
2. DIPEA, DMAP,
Ac_2O, THF***
yield
58 $R^1 = CH_3$ $R^2 = CH_2CH(CH_3)_2$ 11%
59 $R^1 = CH_2CH(CH_3)_2$ $R^2 = CH_2Ph$ 69%
60 $R^1 = CHCH_3CH_2CH_3$ $R^2 = CHCH_3CH_2CH_3$ 27%

* Compound **56** was prepared using $N(Et)_3$, PyBOP, and CH_2Cl_2.
** Compounds **55** and **56** were purified to 85-90% purity and carried on through the last synthetic step.
*** Compound **60** was prepared using Ac_2O, $N(Et)_3$, and THF.

Scheme 8. Synthesis of D-alanine containing tripeptides **58**, **59**, and **60** (with the aid of Ian Armstrong, summer student in the Vederas group).

2.14. Testing Tripeptides as Substrates of LtnJ from CFE of *L. lactis*

As before, a series of reactions were prepared wherein each reaction contained 9 mL CFE in Tris buffer, pH 5.5, one of the tripeptides **43**, **53**, or **54** as a substrate, either NADH and NAD^+ or NADPH and $NADP^+$, and either none or all of the following co-factors; zinc, magnesium, iron, FAD, and FMN. Unlike the previously prepared enzyme reactions, NAD^+ or $NADP^+$ was added to some of the reactions. Recently, the in vitro reconstitution of LctM activity, the enzyme responsible for the dehydration and cyclization reactions in the lantibiotic lacticin 481, indicates that this ATP-dependent enzyme requires ADP in order to dehydrate its substrate.[136] Thus, LtnJ may require $NAD(P)^+$ as well as NAD(P)H in order to exhibit biological activity.

Due to the lack of a known activity assay for LtnJ, the amount of LtnJ in the CFE is unknown. In order to ensure that enough LtnJ is present in the enzyme reactions, the amount of CFE was increased from 4 mL to 9 mL per reaction (equivalent to ~4.5 mg of total protein). As before, the reactions were incubated at 30 °C for 6 hours, lyophilized, and the tripeptides were extracted with methanol. Increasing the amount of CFE used makes analysis by ESMS impossible as there are many components of the CFE that are soluble in methanol and consequently interfere with the analysis. Liquid chromatography coupled with detection via electrospray mass spectrometry (LCMS) is a very powerful method for the analysis of trace components of complex mixtures. Thus a suitable LCMS method was developed for each reaction (see Experimental section 4.2.22.). The conversion of only 0.1% (equivalent to 0.5 nmol) of the dehydroalanine containing

tripeptides **43** and **53** to their subsequent D-alanine containing tripeptides **58** and **59** can be detected using LCMS (Figures 37-42). For the tripeptide **54**, conversion of 1% (equivalent to 5 nmol) to the D-alanine containing tripeptide **60** is detectable (Figures 43-45). Analysis of each prepared enzyme reaction indicates no detectable conversion of the dehydroalanine containing tripeptides **43**, **53**, and **54** to the corresponding D-alanine containing tripeptides **58**, **59**, and **60** (example spectra in Figures 46-48).

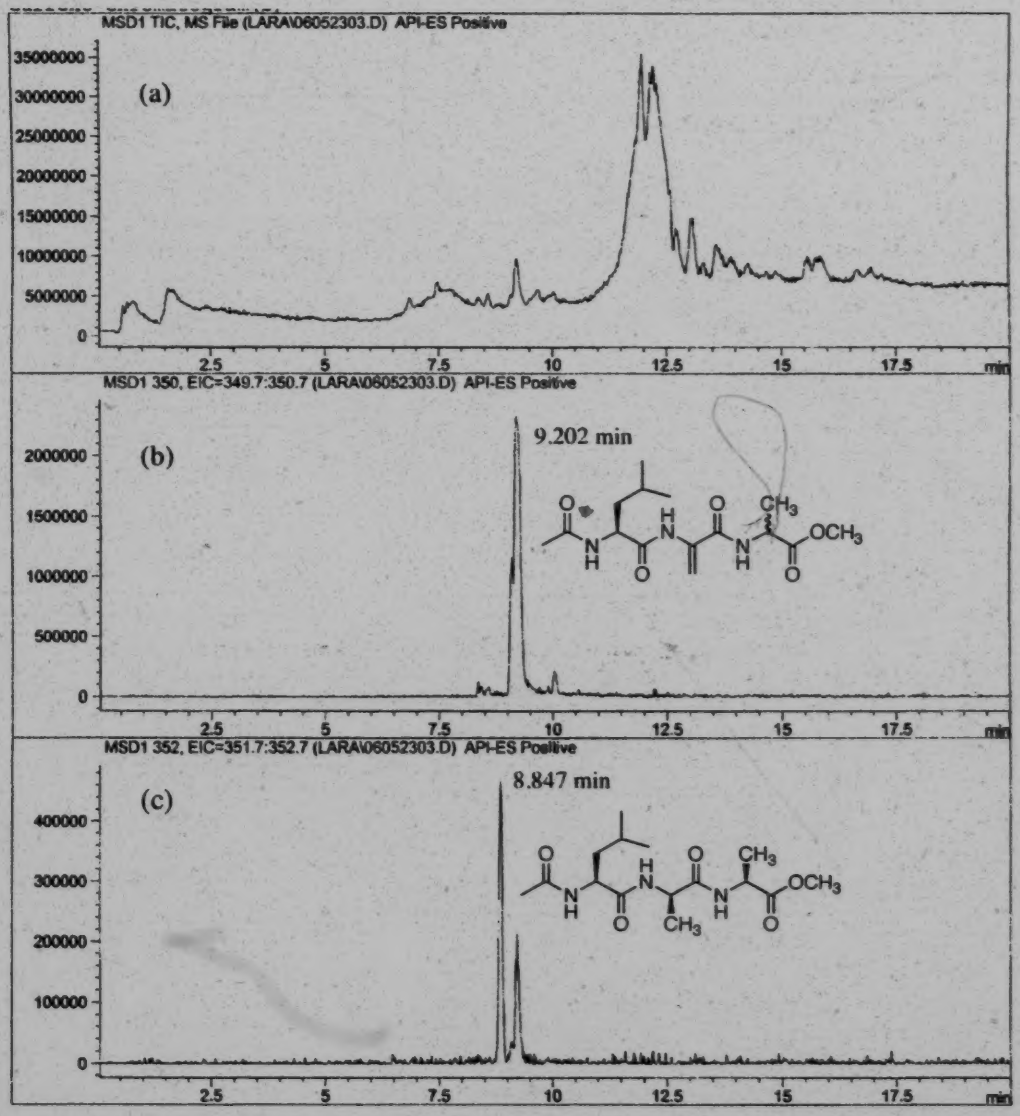


Figure 37. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **43**, 0.5 nmol tripeptide **58**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=350. (c) LCMS trace of single ion selection for m/z=352.

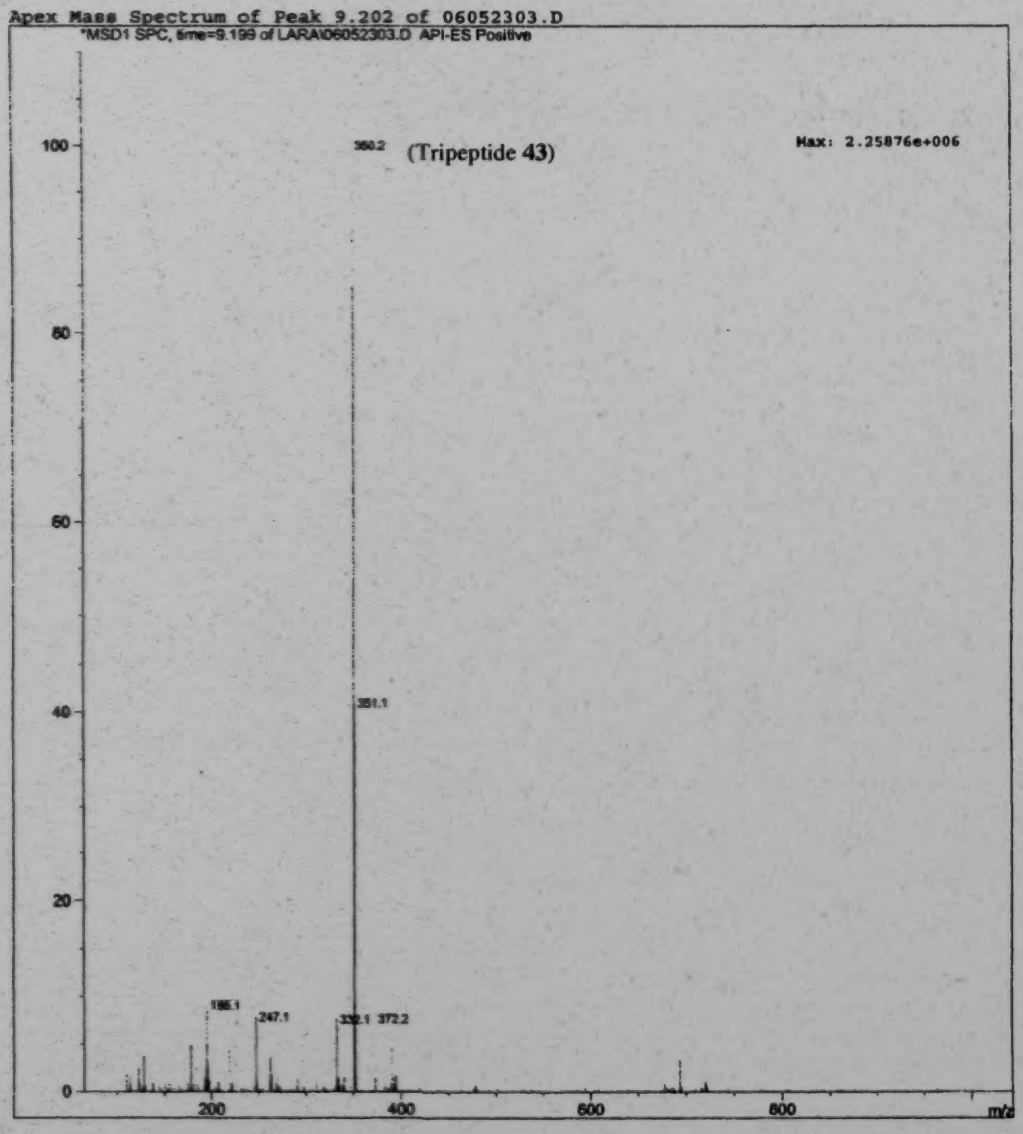


Figure 38. ESMS spectum (120 eV) of LC peak (b) from Figure 37 shows presence of tripeptide **43** (m/z=350).

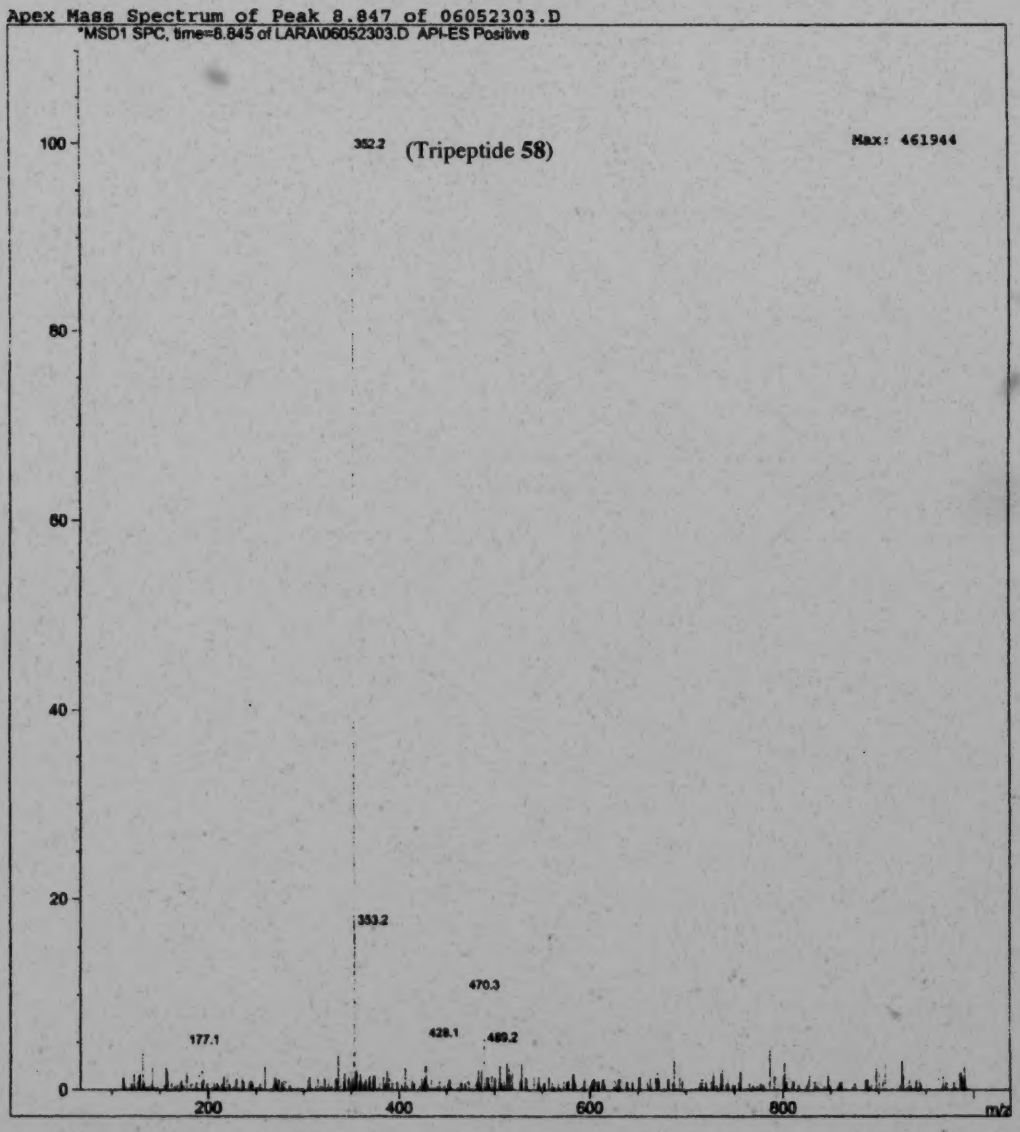


Figure 39. ESMS spectrum (120 eV) of LC peak (c) from Figure 37 shows presence of tripeptide **58** (m/z=352).

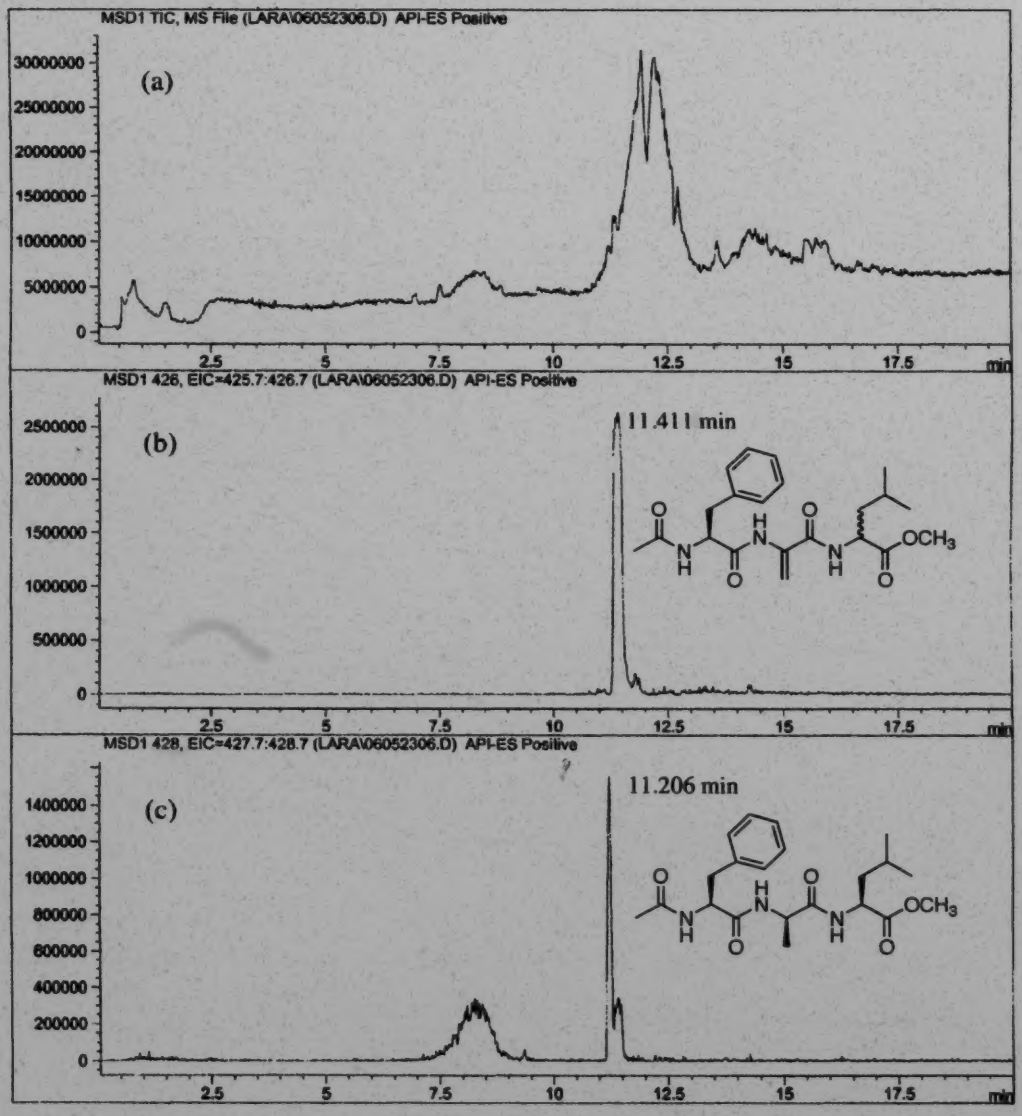


Figure 40. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **53**, 0.5 nmol tripeptide **59**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=426. (c) LCMS trace of single ion selection for m/z=428.

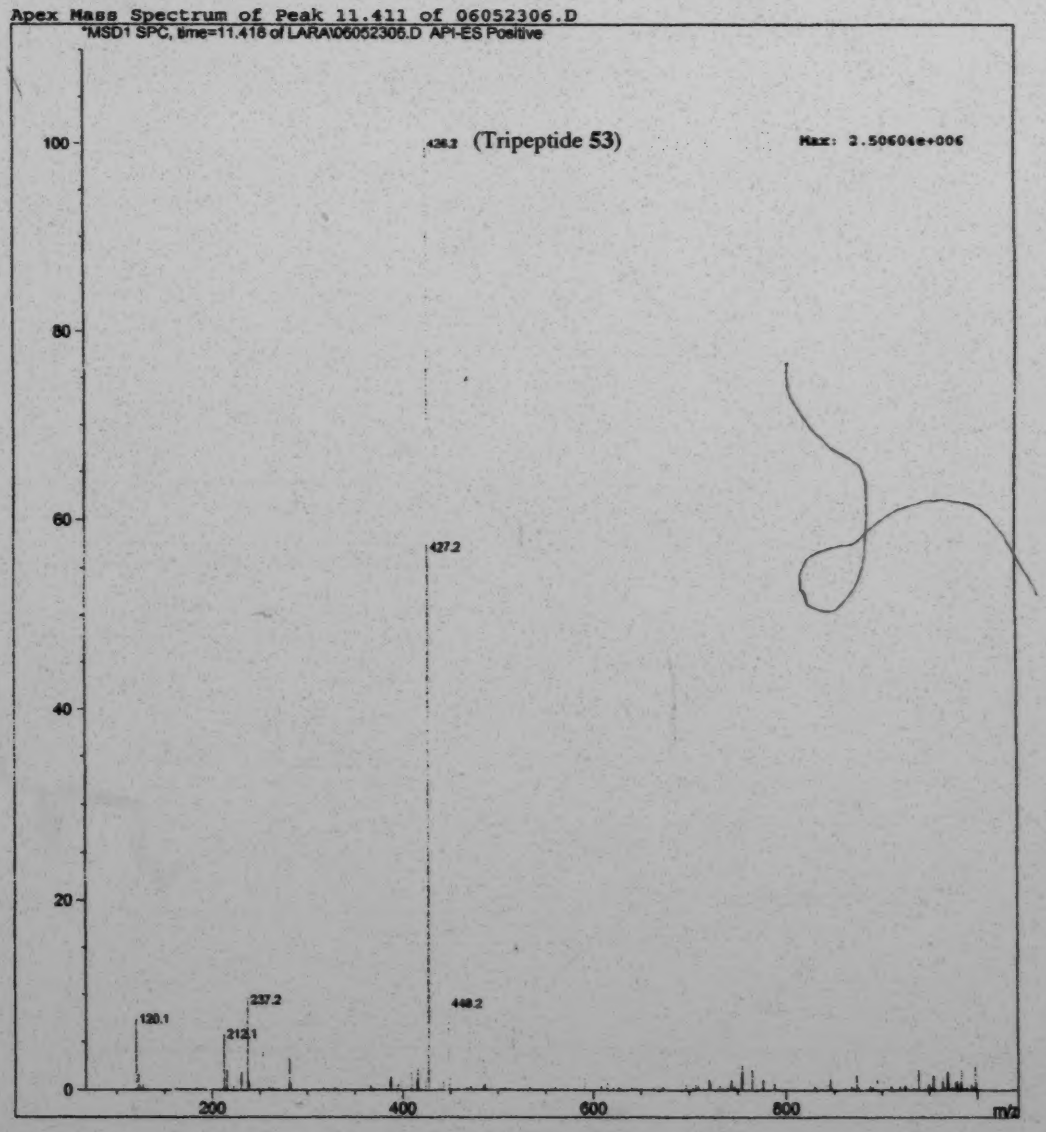


Figure 41. ESMS spectum (120 eV) of LC peak (b) from Figure 40 shows presence of tripeptide **53** (m/z=426).

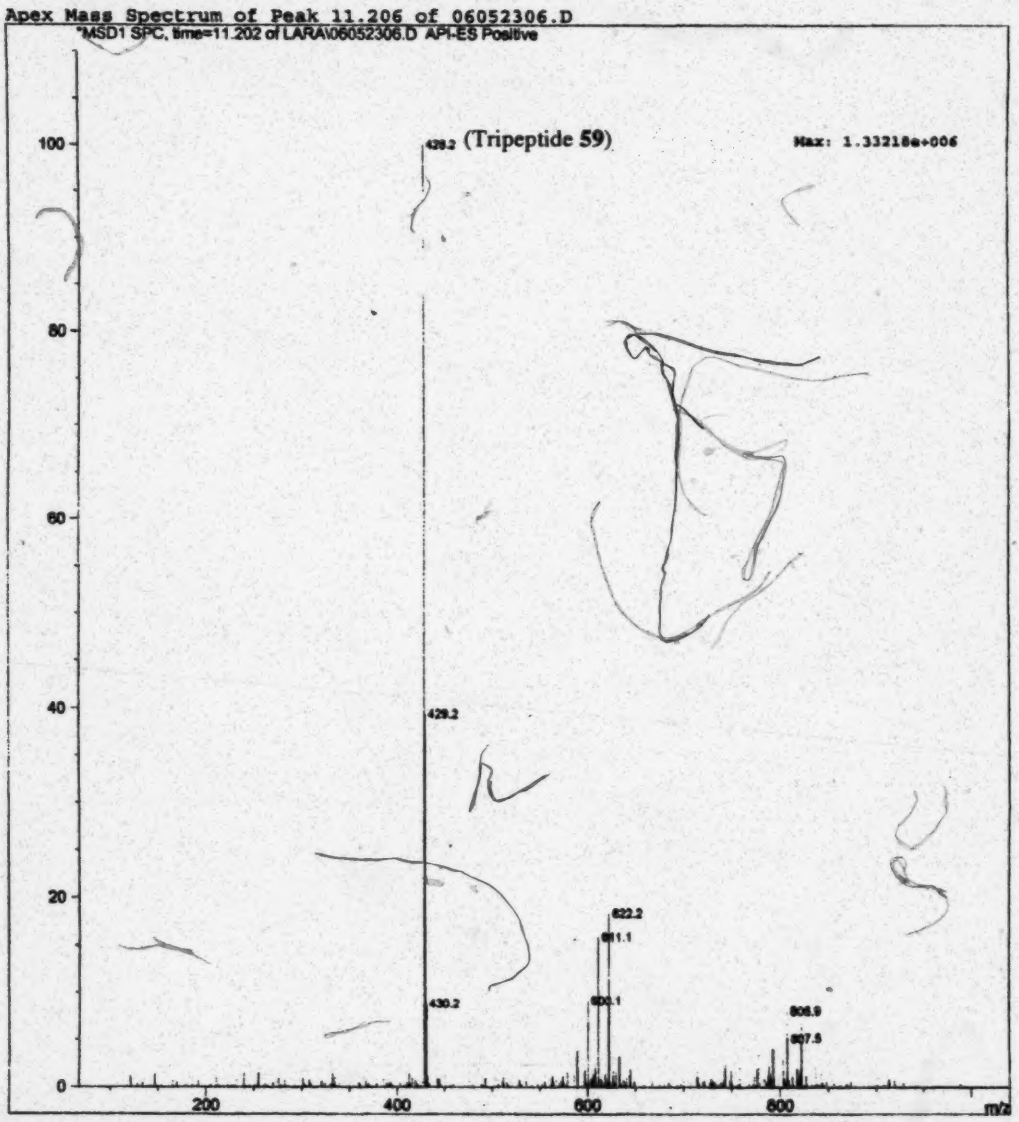


Figure 42. ESMS spectrum (120 eV) of LC peak (c) from Figure 40 shows presence of tripeptide **59** (m/z=428).

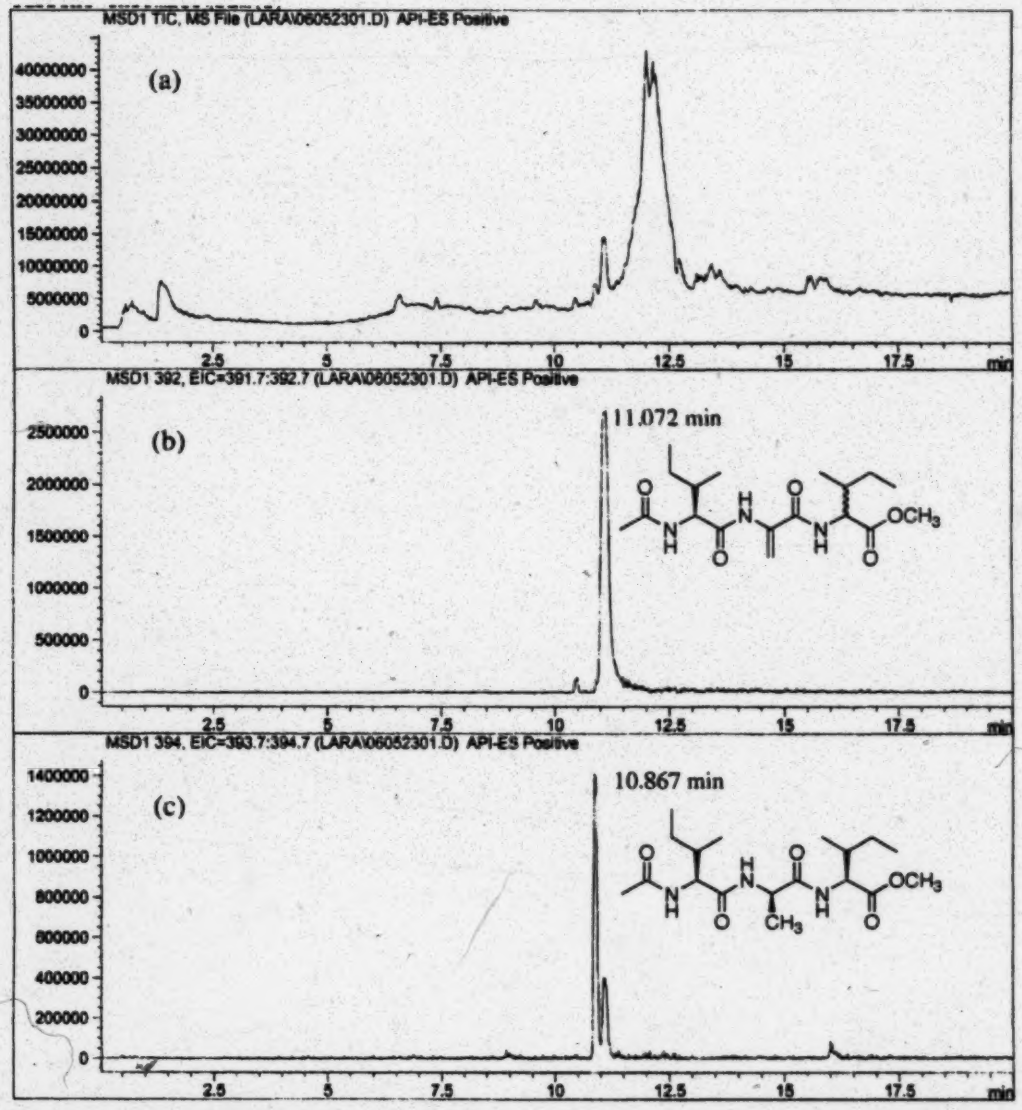


Figure 43. (a) LCMS trace of control assay reaction containing 450 nmol tripeptide **54**, 5 nmol tripeptide **60**, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, and final concentrations of 0.1 μM Zn^{2+}, 0.3 μM Fe^{2+}, 1 μM Mg^{2+}, 1 mM FMN, 1 mM FAD, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=392. (c) LCMS trace of single ion selection for m/z=394.

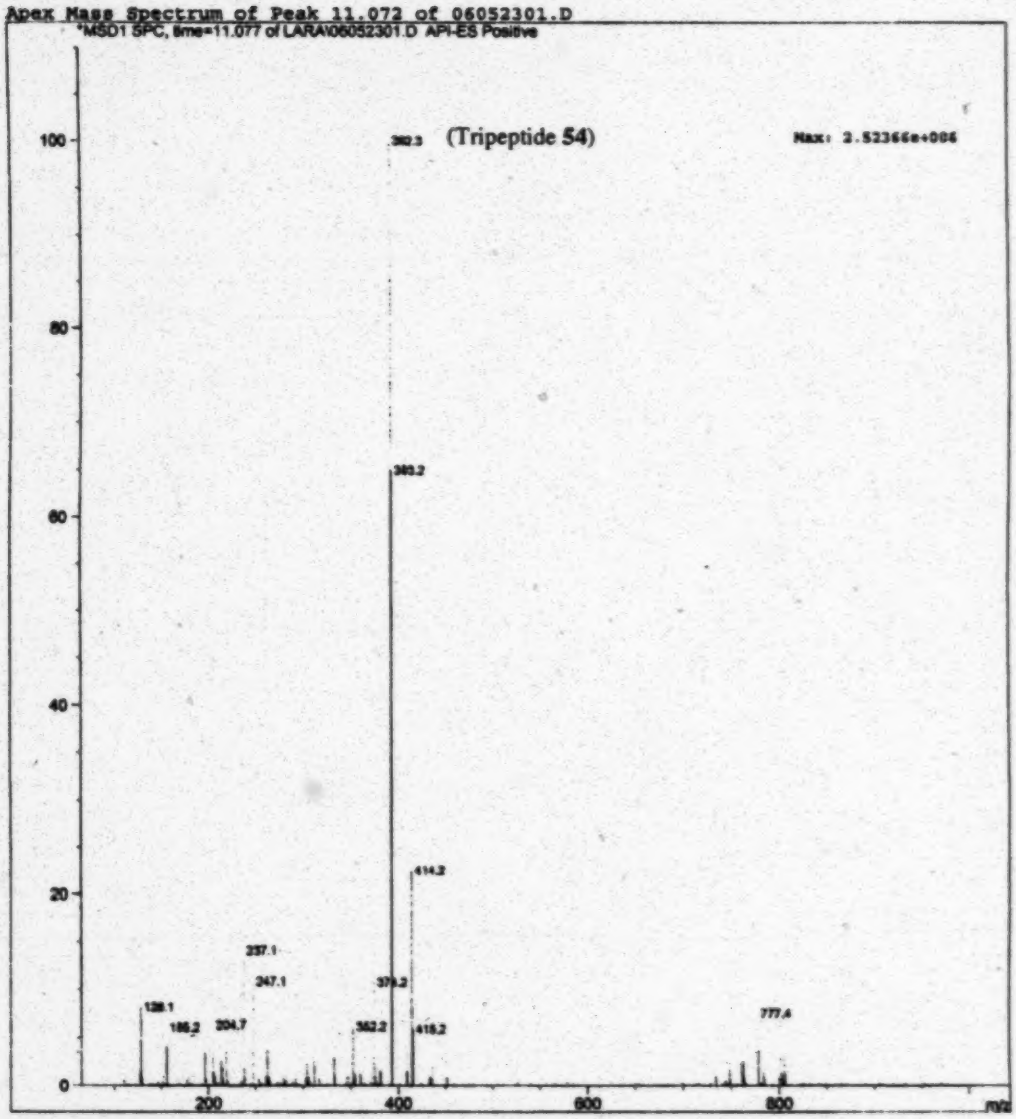


Figure 44. ESMS spectum (120 eV) of LC peak (b) from Figure 43 shows presence of tripeptide **54** (m/z=392).

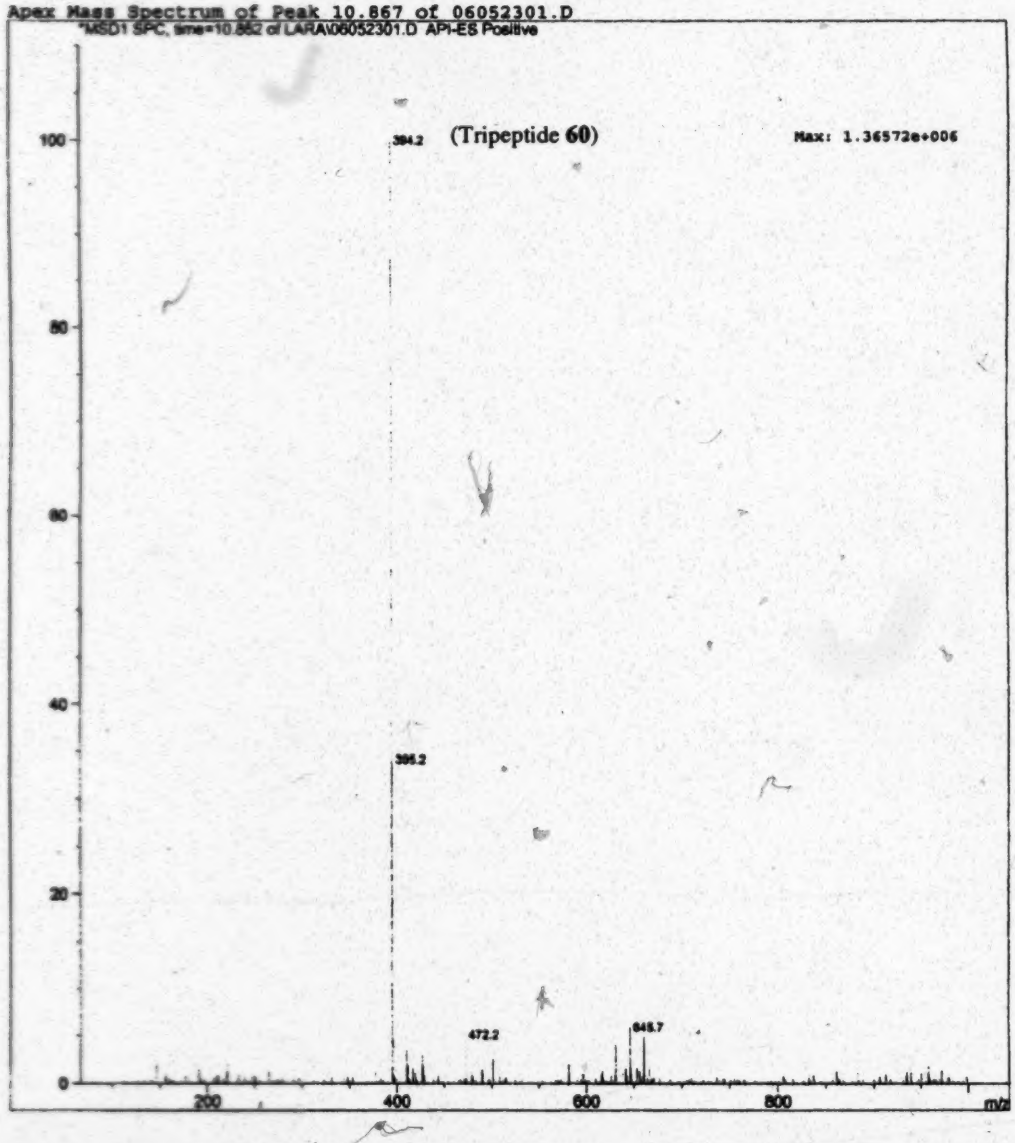


Figure 45. ESMS spectrum (120 eV) of LC peak (c) from Figure 43 shows presence of tripeptide **60** (m/z=394).

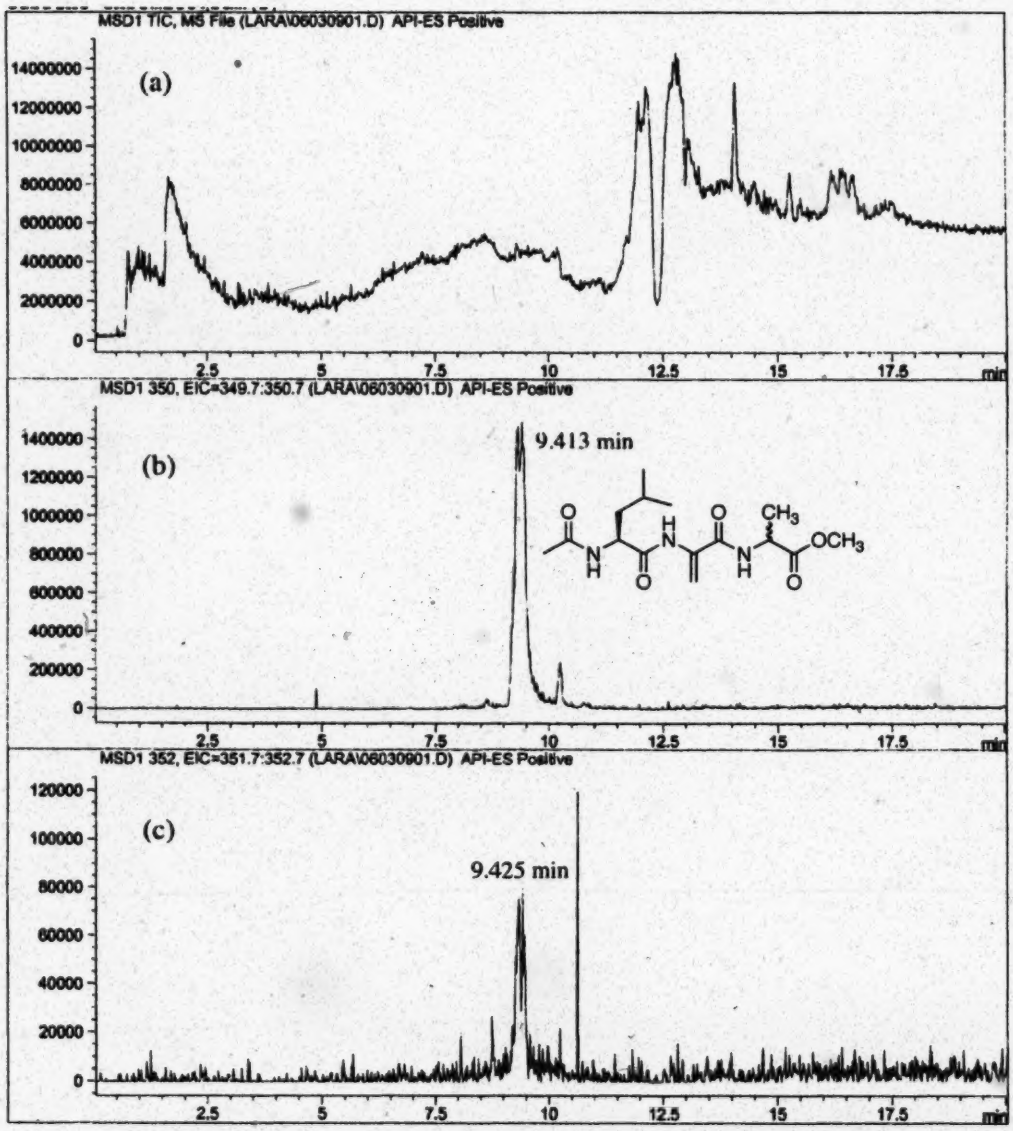


Figure 46. (a) LCMS trace of assay reaction containing 50 nmol tripeptide **43**, 100 nmol NADH, 100 nmol NAD^+, and 9 mL of CFE from *L. lactis* in tris buffer. (b) LCMS trace of single ion selection for m/z=350. (c) LCMS trace of single ion selection for m/z=352.

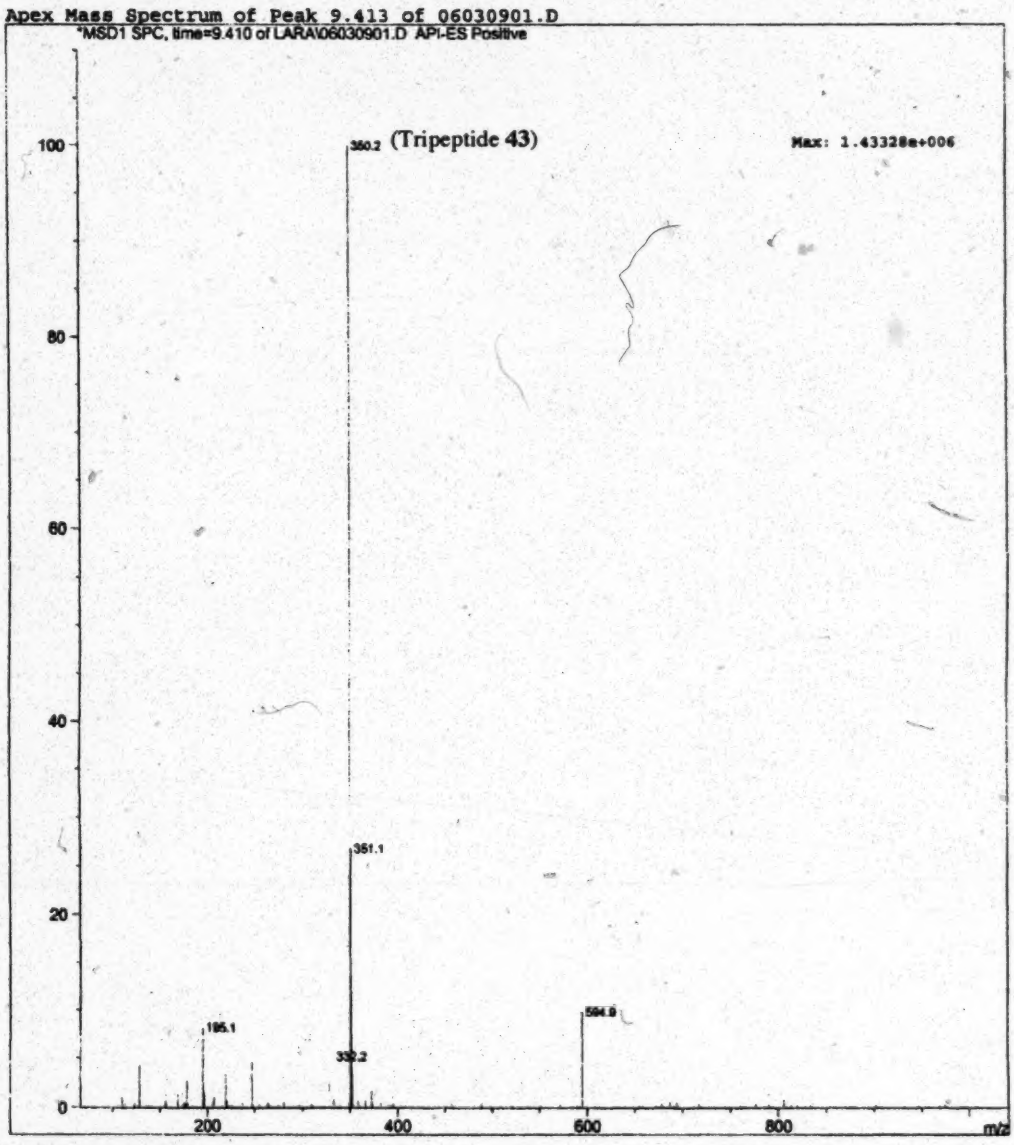


Figure 47. ESMS spectum (120 eV) of LC peak (b) from Figure 46 shows presence of tripeptide **43** (m/z=350).

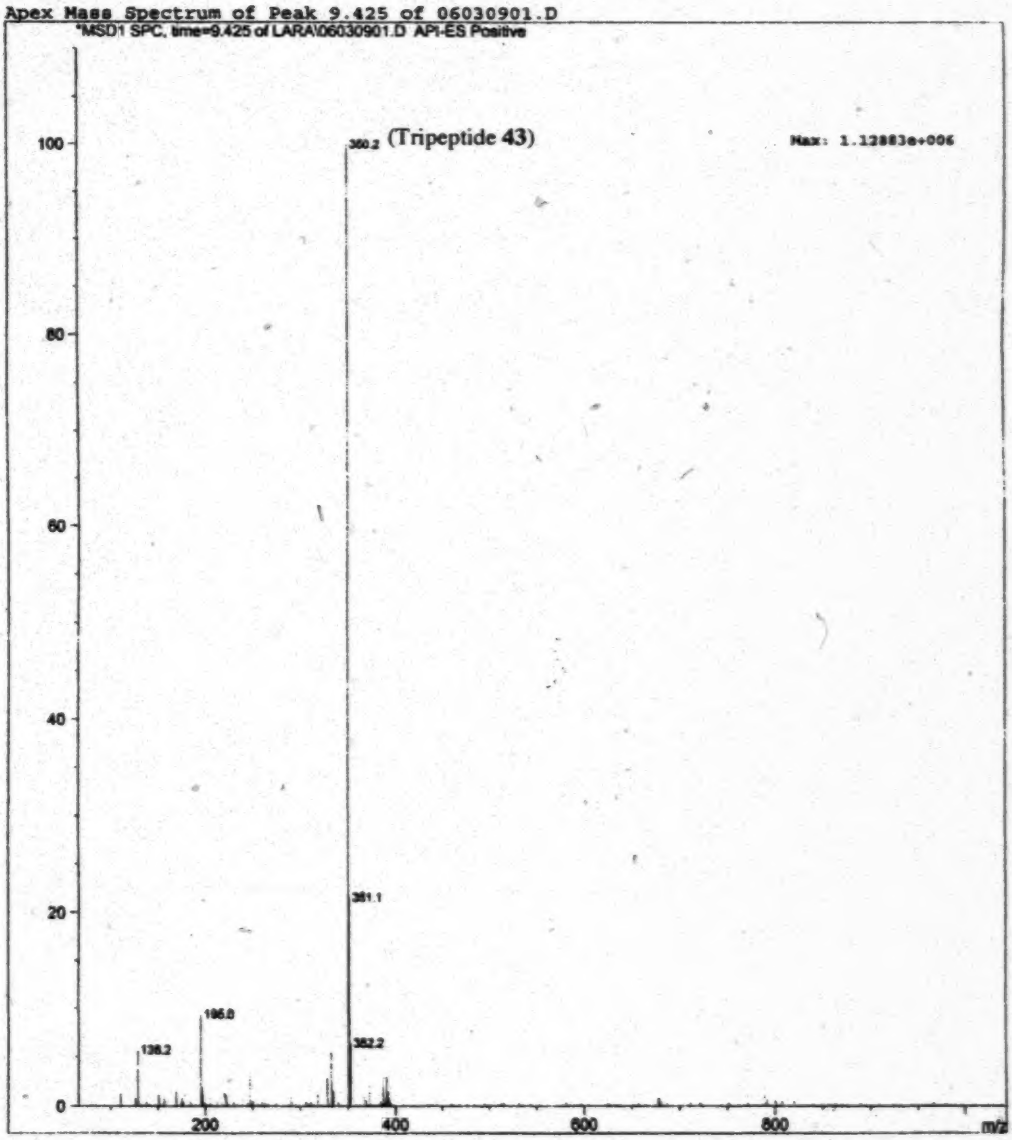


Figure 48. ESMS spectrum (120 eV) of LC peak (c) from Figure 46 shows presence of tripeptide **43** (m/z=350) only. Tripeptide **58** (m/z=352) is not detected.

2.15. Testing Tripeptides as Substrates of LtnJ from *E. coli* K12 ER2507 Containing pLS3

Each of the tripeptides **43**, **53**, and **54** were also tested as substrates of LtnJ obtained from *E. coli* K12 ER2507 containing pLS3. Reactions were prepared and analyzed in a similar fashion as described in section 2.14. and LtnJ in both Tris and phosphate buffer was tested. However, no D-alanine containing tripeptides **58**, **59**, and **60** were detected in any of the reaction mixtures (*data not shown*).

2.16. Isolation of Lacticin 3147 from an *L. lactis* Δ*ltnJ* Mutant

Our collaborator Dr. P. Cotter created a deletion of *ltnJ* in pMRC01, the plasmid in *L. lactis* subsp. *lactis* DPC3147 containing the genes encoding for lacticin 3147 production. This Δ*ltnJ* mutant produces LtnA1 and LtnA2 with 2,3-dehydroalanine residues in place of the D-alanines.[117] Production of both lacticin 3147 peptides is greatly diminished in this mutant compared to the wild-type.

These lacticin 3147 mutants are conceivably substrates for LtnJ. To test this theory, isolation of the lacticin 3147 mutants was initiated. 4 litres of Δ*ltnJ* mutant cells were grown in modified tryptone-yeast broth, the cells were collected and the lacticin 3147 mutants were extracted with 70% IPA. Lyophilization of the extract and analysis by MALDI-TOF mass spectrometry shows the presence of the mutated lacticin 3147 only

when the sample is prepared using a ZipTip® (Figure 49). Because of the extremely low quantity of mutant peptides, purification was not attempted.

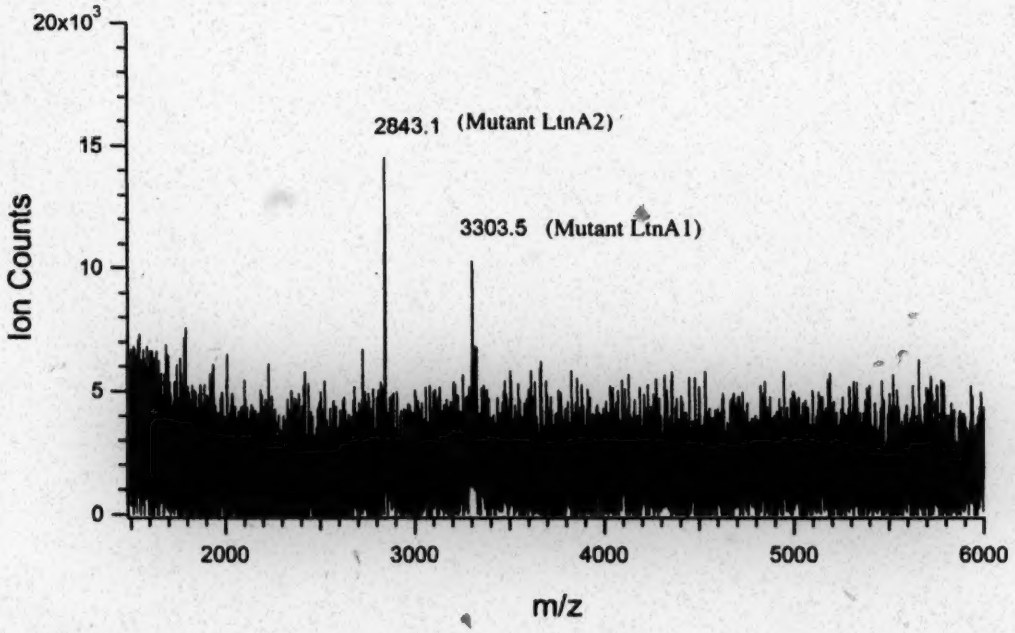


Figure 49. MALDI-TOF mass spectrum of crude lacticin 3147 mutants prepared using a ZipTip®.

2.17. Testing Lacticin 3147 Mutants as Substrates of LtnJ from CFE of *L. lactis*

The crude lacticin 3147 mutants were re-suspended in 10% isopropanol and tested as substrates of LtnJ from the CFE of *L. lactis*. Reactions were prepared and analyzed as described in section 2.10. Each sample was lyophilized and the mutant peptides were extracted with 50 µL IPA. The solvent was removed and the sample reconstituted in 3

µL of water (0.1% TFA). Analysis by MALDI-TOF mass spectrometry revealed only the presence of mutant LtnA1. The signals were very weak and sample preparation using a ZipTip® did not improve the results (*data not shown*). The concentration of the mutant peptides appears to be too low for MALDI-TOF analysis. These results are inconclusive as it is not possible to tell if the lacticin mutants were converted to lacticin 3147.

2.18. Conclusions and Future Directions

Several attempts towards the reconstitution of *in vitro* LtnJ biological activity have been described. The long-term objective is to determine the substrate specificity of LtnJ and use this enzyme for the formation of D-alanine containing peptides. The successful preparation of four LtnJ *E. coli* overexpression strains has been presented. In addition, LtnJ from three different sources was tested for biological activity against three dipeptidyl and three tripeptidyl compounds using several assay conditions. However, biological activity was not observed under any of the described conditions.

To date, *in vitro* reconstitution of enzymatic activity has been described for only a few lantibiotic modification enzymes. Many of these enzymes require the presence of their bacteriocin leader sequence. Thus it is possible that LtnJ also requires the leader sequences of LtnA1 and LtnA2 in order to display its biological activity. Bioinformatic analysis of LtnA1 and LtnA2 leader sequences reveals limited homology (Figure 50).

Even so, determination of the tertiary structure of the pre-lantibiotics bound to their leader peptides may yield greater insight into possible LtnJ recognition sites.

```
LtnA1 leader:        MNKNE-IETQPVTWLEEVSDQNFDEDVFGA
LtnA2 leader:  MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG
                     * **  ** *    **          *
```

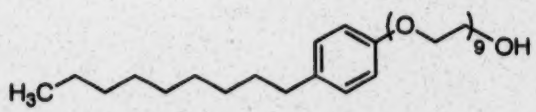
Figure 50. Alignment of the primary structures of the leader sequences of LtnA1 and LtnA2 shows limited homology. Identical residues are indicated with an underlying asterix.

Substrates of LtnJ may consist of peptides containing the leader sequence of LtnA1 or LtnA2 as well as dehydroalanine residues. A defining feature of lantibiotics is the presence of rather large leader sequences, at least when compared to the size of the lantibiotics themselves. Lacticin 3147 is no exception with each leader sequence being comparable in size to LtnA1 and LtnA2 (i.e. LtnA1 leader; 29 residues, LtnA1; 30 residues, LtnA2 leader; 36 residues, LtnA2; 29 residues). This makes synthesis of peptides containing the leader sequence cumbersome. Thus the biological synthesis of such peptides using *E. coli* expression systems may be a more attractive alternative and the design of such peptides is in progress.

CHAPTER 3. ANTIMICROBIAL AGENTS AS SPERMICIDES

3.1. Microbicides and Spermicides

The increase in the transmission of human immunodeficiency virus (HIV) and sexually transmitted infections (STI) has become a global issue. The number of people infected worldwide with HIV is astronomical (~40 million) and there are an estimated 15 000 new HIV infections every day.[137,138] In addition, there are over 340 million STI cases occurring each year world wide.[139] There are also an estimated 133 million unintended pregnancies every year.[139] Presently marketed contraceptive products typically incorporate the membrane surfactant nonoxynol-9 (N-9) (**61**) as the main spermicidal ingredient (Figure 51). Formulations containing 100-150 mg of N-9 are effective at decreasing the likelihood of pregnancy.[140] Although this compound causes the immobilization of sperm, it does not protect against STI.[139] In fact, there is some evidence that it causes lesions on vaginal epithelial cells, which increases the risk of HIV and STI infections.[141] Furthermore, N-9 is known to inactivate lactobacilli, an important bacterium involved in maintaining bacterial homeostasis in the vaginal tract.[142] This results in disturbances in the vaginal microflora and can lead to infections, further increasing the chances of HIV/STI infections. Clearly there is a need for alternative spermicidal products that do not adversely affect vaginal epithelial cells or the vaginal microflora.



61

Figure 51. The structure of nonoxynol-9.

Moreover, spermicidal products that also combat HIV/STI are in great demand. Currently, condoms are the only available product on the market that are capable of preventing unwanted pregnancy and HIV/STI infections when used consistently and correctly.[143] However, women often find it difficult to convince their sexual partners to use such methods. Thus there is a desperate need for new, easy to use, safe and affordable methods that allow women to protect themselves without requiring prior negotiation with their partner.[144,145,146] This need is especially prevalent in developing countries.[140] Furthermore, cultural practices that discourage the use of condoms, as well as the high cost and lack of availability of condoms, all contribute to the need for alternative methods to prevent the transmission of HIV/STI.[140] Microbicides are compounds formulated as gels, films, foams, suppositories, or creams that may prevent the transmission of HIV/STI when inserted into the vagina prior to intercourse. Thus compounds that exhibit both spermicidal and microbicidal activity are obvious solutions to the present issues.

3.2. The Spermicidal Properties of Nisin

Antimicrobial peptides (AMPs) offer a plausible alternative to the existing chemical options as microbicidal agents. Most AMPs are cationic, hydrophobic peptides and exert their microbial properties by binding to the anionic phospholipids present on the outer surface of bacterial cell membranes.[147] By contrast, the anionic phospholipids of mammalian cells are present along the cytoplasmic side of the cell membrane and the outer surface is composed of zwitterionic phospholipids.[148] This explains the low cytotoxicity of many AMPs towards mammalian cells and further implicates AMPs as suitable microbicidal agents.[149]

Nisin is an AMP that exhibits both microbial and spermicidal properties. Aranha *et al.* found nisin to be spermicidal against rat, rabbit, monkey, and human sperm.[94] *In vitro*, the concentration of nisin required for complete immobilization of spermatozoa within 20 seconds is 50 μg/mL in rat, 200 μg/mL in rabbit and 300-400 μg/mL in monkey and human as determined by Sander-Cramer assay.[150] Nisin also exhibits contraceptive capabilities *in vivo*.[95] In rabbits, 1 mg of nisin applied intravaginally prior to mating is sufficient to arrest sperm motility and protect against pregnancy. Furthermore, nisin does not show a cytotoxic effect on red blood cells or vaginal cells at the concentration that is toxic to rabbit spermatozoa.

Gram-negative bacteria are the cause of most STI (Table 3). Although nisin typically does not exert microbial activity against Gram-negative cells at low

concentrations, its affect on this class of bacteria can be induced by the addition of agents that disrupt the Gram-negative outer membrane as discussed in section 1.3.1. Furthermore, concentrations that are required for spermicidal activity (400 μg/mL) are up to 20-fold higher than those required for microbial action against Gram-positive organisms (10-50 μg/mL).[151] At these high concentrations, nisin may have an antimicrobial effect against Gram-negative organisms that cause STI.

Table 3. STI and the causative bacteria.

STI	Causative Bacteria	Type of Bacteria / Cell Wall
Chancroid	*Haemophilus ducreyi*	Gram-negative
Chlamydia	*Chlamydia trachomatis*	Gram-negative
Gonorrhoea	*Neisseria gonorrhoeae*	Gram-negative
Non-gonococcal urethritis	*Ureaplasma urealyticum* *Hycoplasma hominis*	No cell wall No cell wall
Syphilis	*Treponema pallidum*	Gram-negative
Bacterial Vaginosis	*Bacteroides* *Veillonella* *Fusobacterium* *Mobiluncus* *Mycoplasma hominus* *Ureaplasma urealyticum* *Eubacterium* *Peptostreptococcus* *Streptococcus viridans* *Gardnerella vaginalis*	Gram-negative Gram-negative Gram-negative Gram-negative with Gram-positive cell wall No cell wall No cell wall Gram-positive Gram-positive Gram-positive Gram-positive
Donovanosis	*Calymmatobacterium granulomatis*	Gram-negative

3.3. The Testing of Bacteriocins as Spermicides

The recent discovery of the spermicidal properties of nisin provides precedent for the testing of other bacteriocins as spermicides. Lacticin 3147 is a lantibiotic closely related to nisin and thus its effect on spermatozoa was evaluated. Initially, the immobilization of cryogenically preserved human spermatozoa was attempted with nisin and lacticin 3147 using the Sander-Cramer assay.[150] The sperm samples were obtained from the Fertility & Women's Endocrine clinic at the Royal Alexandra Hospital (Edmonton, AB). The results indicate that both nisin and lacticin 3147 have no effect on the motility of cryogenically preserved human sperm at concentrations of 400 μg/mL for up to 5 minutes. This is surprising since nisin has been previously shown to completely immobilize human spermatozoa in 20 seconds at this concentration. A modified Ackerman's cryoprotective medium was used to store the semen samples and is made from a glycerol-egg-yolk-citrate base. Preserved semen samples are prepared by mixing the sample with the cryoprotective medium in a 1:1 ratio. Thus the activity of both bacteriocins in the cryopreserving medium was questioned. Nisin and lacticin 3147 were tested for their inhibition of cell growth of the indicator organism *L. lactis* subsp. *cremoris* HP both with and without the human cryopreservative by well-plate diffusion assay. The results show no significant reduction in biological activity of either lantibiotic against *L. lactis* subsp. *cremoris* HP when tested with the preservative (Figure 52). However, this indicates that the mechanism of killing is different for sperm cells than for bacterial cells, an expected result as sperm cells do not contain peptidoglycan or lipid II.

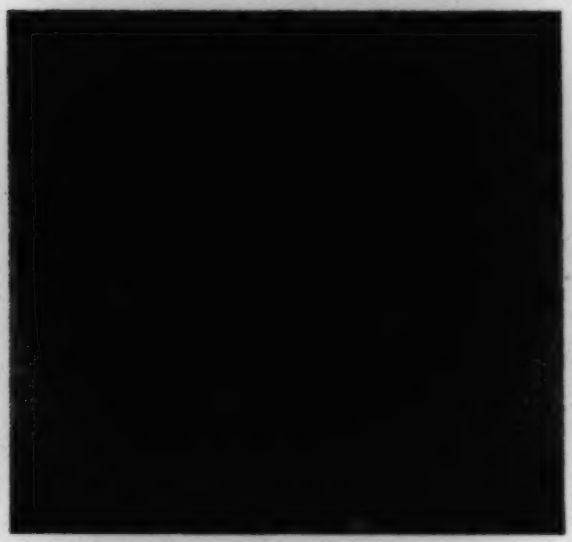


Figure 52. Well-plate diffusion assay of nisin and lacticin 3147 against *L. lactis* subsp. *cremoris* HP with and without human semen cryopreservative. 1; *LtnA1 alone*, 2; *LtnA2 alone*, 3; *nisin alone*, 4; *cryopreservative alone*, 5; *LtnA1 + cryopreservative*, 6; *LtnA2 + cryopreservative*, 7; *nisin + cryopreservative.*

Because freshly collected human semen was not available without an extensive approval process and special laboratory facilities, semen from other mammalian species was investigated. Freshly collected boar semen was available from the Swine Research and Technology Center at the University of Alberta Farm. Furthermore, horse/pony and bull semen was available from two veterinary clinics in the surrounding Edmonton area. This allowed exploration of the ability of nisin and lacticin 3147 to immobilize mammalian sperm. In addition, subtilosin A, a bacteriocin with interesting structural features, was also evaluated for its spermicidal properties. Dose and time-dependent effects of each peptide were determined *in vitro* by Sander-Cramer assay in duplicate. For bovine and horse/pony sperm tests, 800 μg/mL of each bacteriocin was tested as this is the maximum amount that can be solubilized in the testing medium used (10% DMSO

in Ringer's solution). Boar sperm was found to be completely incompatible with a range of organic solvents (Table 4). Thus 200 μg/mL of each bacteriocin was tested as this is the maximum amount that can be solubilized in Ringer's solution without the aid of any organic solvents. For all the bacteriocins tested, duplicate results were obtained and all values displayed are averaged values from the two experiments. Each duplicate experiment yielded results within one minute of each other for the time to completely immobilize sperm. Each sample showing arrest of sperm motility was further analyzed using eosin-nigrosin staining, a live/dead sperm test, and viewing at 400x magnification.[152] Only samples indicating dead sperm in all of 10 consecutive fields were recorded as "pass." Furthermore, glucose was used in a sperm revival test to ensure that sperm could not regain motility.[94,95]

Table 4. Stability of boar sperm in various solvents.

Solvent	Time for Boar Sperm to Die (min)
100% Ringer's solution (control)	120
90% Ringer's solution + 10% DMSO	10
90% Ringer's solution + 10% Ethylene glycol	60
90% Ringer's solution + 10% 1,4-Dioxane	8
90% Ringer's solution + 10% Trifluoroethanol	0.5
90% Ringer's solution + 10% Acetonitrile	5
90% Ringer's solution + 10% Glycerol	25
90% Ringer's solution + 10% Isopropyl alcohol	8

The bacteriocins exhibit similar effects on bovine and horse/pony spermatozoa (Figures 53 and 54). For both sperm samples, LtnA1 is the least effective spermicide, taking an average of 16.6 and 12.7 minutes to completely immobilize bovine and

horse/pony spermatozoa, respectively. Interestingly, LtnA2 is a considerably better spermicide than LtnA1 and shows similar sperm mobility inhibitory effects as nisin. Subtilosin A is a moderately potent spermicide and immobilizes the sperm cells faster than nisin, LtnA1, and LtnA2. In order for compounds to be useful contraceptives, they generally must be capable of immobilizing sperm within 30 seconds. This is because sperm are able to migrate into the cervix and upper reproductive tract extremely quickly. The only bacteriocin capable of immobilizing bovine and horse/pony sperm this rapidly is lacticin 3147 when both peptides are used together.

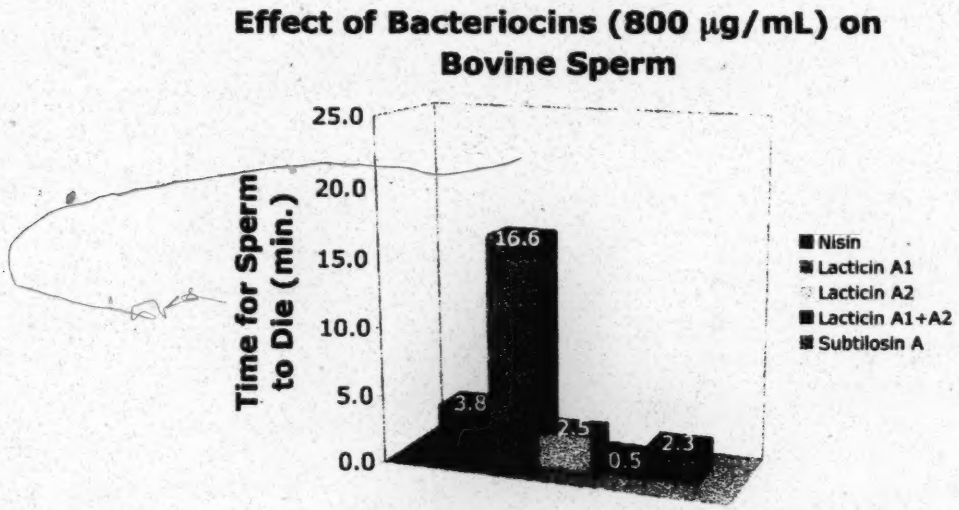


Figure 53. The effect of 800 µg/mL of nisin, lacticin 3147, and subtilosin A on bovine sperm.

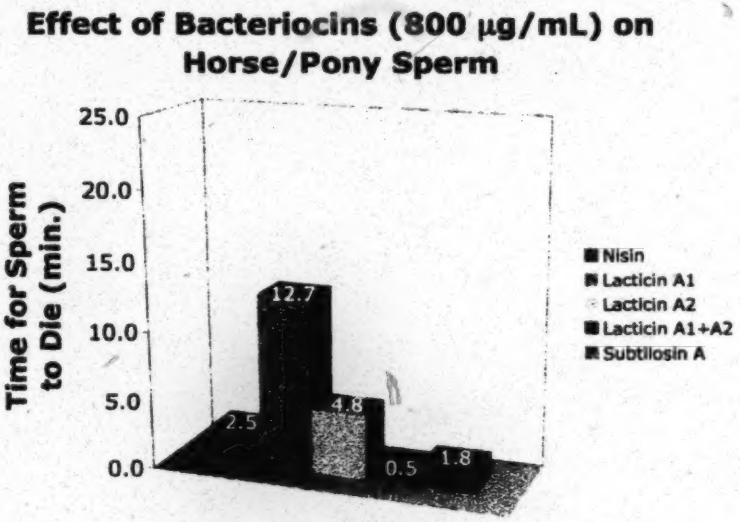


Figure 54. The effect of 800 µg/mL of nisin, lacticin 3147, and subtilosin A on horse/pony sperm.

In contrast, boar sperm react quite differently to the bacteriocins with nisin showing the poorest inhibition of sperm motility (Figure 55). Nisin at a concentration of 200 µg/mL is virtually ineffective at immobilizing pig sperm. LtnA1 and LtnA2, on the other hand, are moderate boar spermicides when used individually, but when tested together, they exhibit excellent spermicidal activity. Subtilosin A yields identical activity as lacticin 3147 and both immobilize boar sperm within 30 seconds.

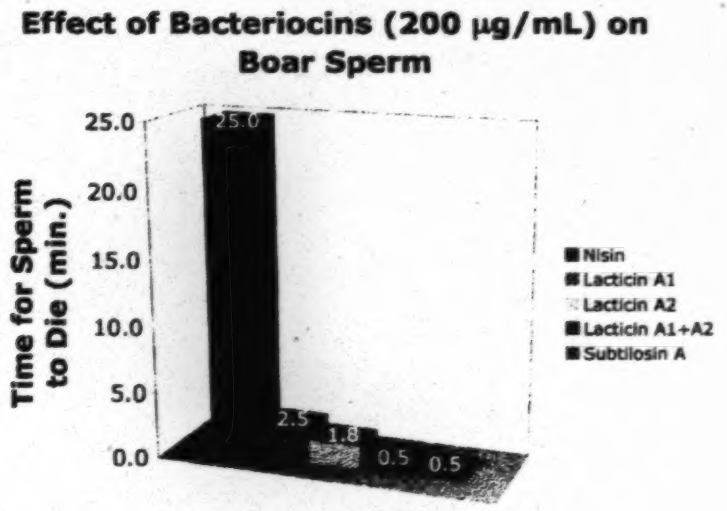


Figure 55. The effect of 200 µg/mL of nisin, lacticin 3147, and subtilosin A on boar sperm.

In order to compare the activities of lacticin 3147 and subtilosin A with those previously published for nisin, the bacteriocins had to be tested against either rat, rabbit, or monkey sperm. Rat sperm samples were available from Dr. Susan Jacobs-Kaufman's laboratory in the Department of Physiology & Medicine at the University of Alberta. The samples were obtained by surgical extraction from the cauda epididymis in a modified procedure from that described by Klinefelter *et al.*[153] The cauda epididymis was surgically exposed from an anesthetized rat, placed in a dish containing the testing medium, and small incisions were made in the sperm filled tubules. The sperm were allowed to disperse into the medium and then were stored at 37 °C until needed. Initial attempts to obtain live sperm using Ringer's medium were unsuccessful as this medium did not contain sufficient nutrients. Live sperm were obtained using a buffered Hanks' Balanced Salt Solution supplemented with glucose and cell stabilizing agents, described

by Slott *et al.*, as the collection and testing medium.[154] As with bull, horse/pony, and boar sperm, the bacteriocins were tested against rat sperm using the Sander-Cramer assay. Rat sperm are also incompatible with organic solvents and so 200 μg/mL of each bacteriocin was tested.

Interestingly, 200 μg of each bacteriocin has a moderate or strong killing effect on rat sperm (Figure 56). Once again, the lacticin 3147 peptides show the most potent activity only when tested in combination. In contrast to published results, nisin takes 3 minutes to kill rat sperm at a concentration of 200 μg/mL. Aranha *et al.* found nisin to completely immobilize rat sperm in 20 seconds at a concentration of 50 μg/mL.[94] It is unclear as to why the results presented in this study do not match those obtained by Aranha *et al.*, although a few experimental differences may contribute to the large observed discrepancy. All the rats used in our study were of the Long-Evans type whereas Aranha *et al.* used Holtzman strain rats. In addition, Aranha *et al.* collected the rat sperm from the cauda epididymis after autopsy whereas we collected from anesthetized rats. Furthermore, we found that the use of Ringer's buffer as the collection and testing medium did not yield live sperm, yet Aranha *et al.* were able to use physiological saline for their collection and testing. Lastly, the method of purification of nisin may further contribute to its spermicidal activity. Nisin is purchased from Sigma as a 2.5% mixture contaminated with milk proteins and salts. We purified nisin via RP-HPLC and confirmed the presence of a pure sample using MALDI-TOF mass spectrometry. Aranha *et al.* purified nisin using a gel filtration column and pooled the fractions containing nisin as determined by SDS-PAGE. Presumably, the purification

technique used by Aranha *et al.* yields reasonably pure nisin, but without analysis of the SDS-PAGE gels it is impossible to know the precise level of purity obtained.

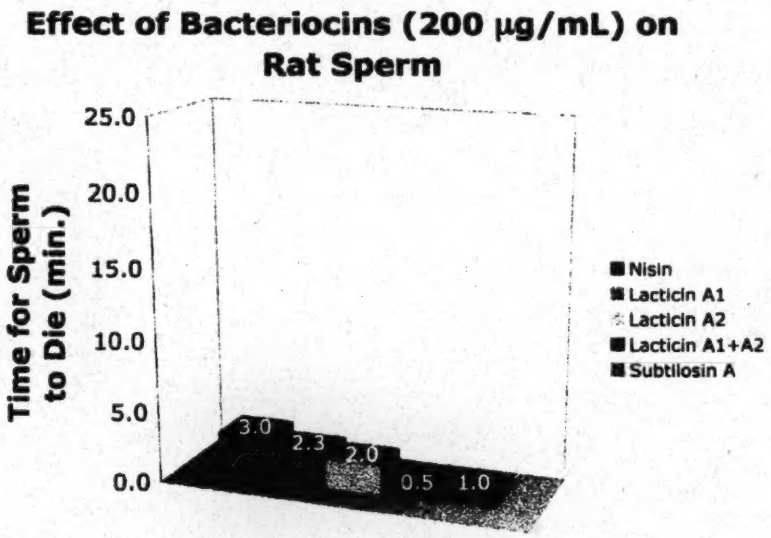


Figure 56. The effect of 200 µg/mL of nisin, lacticin 3147, and subtilosin A on rat sperm.

Lacticin 3147 shows the highest spermicidal activity for all animals tested and was analyzed further to determine its minimum inhibitory concentrations (Figure 57). Interestingly, 200 µg/mL of lacticin 3147 (i.e. 100 µg/mL of LtnA1 and LtnA2 in combination) instantly kills bovine, horse/pony, and rat sperm. As little as 50 µg/mL of lacticin 3147 is able to kill boar sperm. For all animals tested, the lacticin 3147 peptides are more potent spermicides when tested together than when tested individually. This suggests that the spermicidal activity of lacticin 3147 is not simple a surfactant effect and that lacticin 3147 may have a specific spermicidal mode of action.

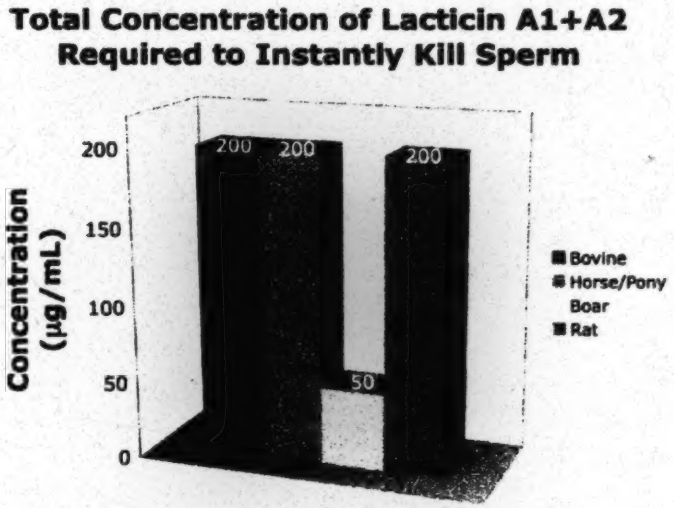


Figure 57. The minimum inhibitory concentration of lacticin 3147 required to instantly kill bovine, horse/pony, boar, and rat sperm.

3.4. Conclusions and Future Directions

As discussed in sections 1.3.1 and 1.3.2, nisin and lacticin 3147 kill their target bacterial cells via the formation of pores in the cell membranes. Both bacteriocins use lipid II as a docking molecule to form these pores. Obviously, sperm cells do not contain lipid II as they do not contain peptidoglycan. But nisin is a cationic peptide and as such can interact with the negatively charged outer membrane of bacterial cells in order to induce pores. Similarily, sperm plasma membranes contain high concentrations of anionic phospholipids such as phophatidylglycerol and phosphatidylserine. Thus nisin may be attracted to sperm cells via ionic forces and subsequently form pores in their plasma membranes. This also explains the lack of cytotoxicity of nisin towards red blood

cells, which contain zwitterionic species in their outer membrane, and vaginal cells, which have a low content of negatively charged phospholipids in their outer membrane.[95,148,149] This may also be true for LtnA2, a positively charged peptide, and explains its higher activity towards bull and horse/pony sperm cells as compared to its neutral partner LtnA1. But this hypothesis does not explain the results obtained for subtilosin A, a neutral bacteriocin which shows higher spermicidal activity than nisin, LtnA1, or LtnA2. As the microbial mode of action of subtilosin A has not yet been elucidated, it is difficult to speculate on its spermicidal mechanism. This theory also does not explain the increased spermicidal activity of LtnA1 and LtnA2 when the peptides are used concomitantly.

Reddy *et al.* hypothesize that nisin inhibits the activity of the mitochondrial enzyme succinate dehydrogenase (SDH), leading to the inhibition of sperm motility.[95] In a (3-[4-5-dimethylthiazol-2-4]-2,5-diphenyltetrazolium bromide (MTT) assay, MTT is reduced by SDH to give an insoluble, dark blue formazan product. Rabbit sperm cells were first treated with nisin, then with MTT. Analysis of the resulting solution shows low levels of formazan in the nisin treated sperm cells, indicating diminished levels of SDH. It is possible that lacticin 3147 and subtilosin A are also capable of inhibiting this enzyme, explaining the loss of sperm motility of cells treated with these bacteriocins.

There is a need for dual-acting products that exhibit contraceptive as well as microbicidal properties. Bacteriocins are attractive agents for the development of such products. This study demonstrates the spermicidal activity of nisin, lacticin 3147, and

subtilosin A against bovine, horse/pony, boar, and rat sperm. In particular, lacticin 3147 is a potent inhibitor of sperm motility in each of the species tested only when both peptides are used in concert. Nisin has previously been shown to be nontoxic to humans[155] and shows no cytotoxicity to red blood cells and vaginal epithelial cells. As lacticin 3147 is a lantibiotic closely related to nisin and is produced by a food-grade bacterium, this bacteriocin may also exhibit such properties. Furthermore, effective spermicides/microbicides must be able to withstand a broad pH range. The vaginal pH is around 4 and rises to 8 after the deposition of semen. Nisin is unstable at basic pH and thus may not be as effective as lacticin 3147, which is stable in this pH range.

In order to fully evaluate the potential of lacticin 3147 as a spermicide/microbicide, its activity against Gram-negative bacteria must be realized. In addition, its toxicity, or lack there of, to mammalian cells must also be determined. Currently, experiments are in progress to evaluate such parameters. Before bacteriocins can truly be used as contraceptive agents, their mechanism of spermicidal action must be determined. In particular, the mechanism of antimicrobial action of subtilosin A is completely unknown. Determination of this may reveal important information about its spermicidal mechanism as it is an uncharged peptide. This information may be particularly useful since subtilosin A is not a member of the lantibiotics and thus may shed light on the spermicidal mode of action of nisin and lacticin 3147. In our laboratory, experiments are underway to determine the mechanism of subtilosin A activity. Furthermore, the low solubility of bacteriocins in Ringer's solution without the aid of

organic solvents creates new challenges for the development of appropriate cream, foam or jelly formulations.

CHAPTER 4. EXPERIMENTAL PROCEDURES

4.1. Synthesis of Dipeptides and Tripeptides

GENERAL METHODOLOGIES

4.1.1. Reagents, Solvents, and Solutions

All reactions involving air or moisture sensitive reactants were done under a positive pressure of dry argon using oven-dried glassware. Reactions performed at room temperature refers to 25 °C. All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. For anhydrous reactions, solvents were dried according to Perrin *et al.*[156] and Vogel.[157] Acetonitrile, dichloromethane, and methanol were distilled over calcium hydride under an argon atmosphere. Deionized water was obtained from a Milli-Q reagent water system (Millipore Co., Milford, MA). Removal of solvent was performed under reduced pressure below 40 °C using a Büchi rotary evaporator, followed by evacuation (< 0.1 mm Hg) to constant sample weight. Unless otherwise specified, solutions of HCl and NaOH refer to aqueous solutions. Brine refers to a saturated aqueous solution of NaCl.

4.1.2. Purification Techniques

All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using glass plates with a UV fluorescent indicator (normal

SiO_2, Merck 60 F_{254}; reversed-phase, Merck RP-8 and RP-18 F_{254}). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; iodine staining; phosphomolybdic acid/ceric sulfate/sulfuric acid/H_2O (10 g:1.25 g:12 mL:238 mL) spray; and Ninhydrin/methanol (1 g:100 mL) spray. Flash chromatography was performed according to the method of Still *et al.*[158] using Merck type 60, 230-400 mesh silica gel.

4.1.3. Instrumentation for Compound Characterization

Optical rotations were measured on a Perkin Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are reported in units of 10^{-1} deg cm^2 g^{-1}. All optical rotations reported were referenced against air and were measured at the sodium D line and values quoted are valid within ±1°. Infrared spectra (IR) were recorded on a Nicolet Magna 750 or a 20SX FT-IR spectrometer. Mass spectra (MS) were recorded on a Kratos AEIMS-50 high resolution (HRMS), 0.5% solution of formic acid in MeCN:H_2O/1:1) instrument.

Nuclear magnetic resonance (NMR) spectra were obtained on Inova Varian 300, 400, and 500 MHz spectrometers. ^1H NMR chemical shifts are reported in parts per million (ppm) using the residual proton resonance of solvents as reference: $CDCl_3$ δ 7.24 and $(CD_3)_2CO$ δ 2.04. ^{13}C NMR chemical shifts are reported relative to $CDCl_3$ δ 77.0 and $(CD_3)_2CO$ δ 39.5. Selective homonuclear decoupling, attached proton test (APT) was occasionally used for signal assignments. ^1H NMR data are reported in the following

order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dq, doublet of quartets; and m, multiplet), number of protons, coupling constant(s) (*J*) in Hertz (Hz), and assignment. When appropriate, the multiplicity is preceded by br, indicating that the signal was broad.

GENERAL PROCEDURES FOR COMPOUND PREPARATIONS

4.1.4. General Procedure for Preparation of Dehydroalanine Containing Dipeptides (24-26)

4-Methyl morpholine (1 mol eq.), isobutyl chloroformate (1 mol eq.), and the L-amino acid methyl ester hydrochloride salt (1 mol eq.) were added successively to a solution of 2-acetamidoacrylic acid (0.9 mol eq.) in THF (10 ml per mmol L-amino acid methyl ester hydrochloride salt) at room temperature. The reaction solution was left to stir at room temperature for 1 h 15 min before being filtered, and the filtrate was concentrated under vacuum to give the crude product. Purification was accomplished via flash chromatography (SiO_2, 100% EtOAc for compound **24**, 1:2 / hexane: EtOAc for compound **25**, 1:1 / hexane: EtOAc for compound **26**).

4.1.5. General Procedure for Preparation of Protected D-Alanine Containing Dipeptides (31-33)

The Boc-protected amino acid (1 mol eq.) was added to a slurry of the appropriate amino acid methyl ester hydrochloride salt (1 mol eq.) in dichloromethane (10 ml per mmol Boc-protected amino acid) at room temperature. The resulting reaction solution

was cooled to 0-5 °C and $N(Et)_3$ (2 mol eq.), EDC·HCl (1 mol eq.), and HOBt (1 mol eq.) were added sequentially. The mixture was left to warm to room temperature over 24 h. Removal of the solvent under reduced pressure afforded a residue that was purified by column chromatography (SiO_2, 1:1 / hexane: EtOAc for compound **31**, 100% EtOAc for compounds **32** and **33**).

4.1.6. General Procedure for Preparation of D-Alanine Containing Dipeptides (34-36, 60)

TFA (1.5 ml per mmol Boc-protected dipeptide) was added drop-wise to a solution of the appropriate Boc-protected dipeptide (1 mol eq.) in dichloromethane (20 ml per mmol Boc-protected dipeptide) at room temperature. The reaction solution was left to stir at room temperature for 2.5 hr before it was concentrated under reduced pressure and the resulting residue was evaporated with $(Et)_2O$ (3-4 times, 15 ml per mmol dipeptide). The product was left on the high vacuum overnight. The TFA salt of the dipeptide was dissolved in THF (20 ml per mmol dipeptide) and acetic anhydride (10 mol eq.) and $N(Et)_3$ (5 mol eq.) were added at room temperature. The reaction solution was then heated to 50 °C and left to stir overnight at this temperature. Upon cooling, the solution was concentrated under reduced pressure to give the crude product, which was purified via flash chromatography for compounds **34-36** (SiO_2, 1:9 / EtOH:EtOAc). Compound **60** was purified via recrystalization using methanol.

4.1.7. General Procedure for Preparation of Protected Serine Containing Dipeptides (38, 45, 46)

The amino acid ester hydrochloride (1 mol eq.) was dissolved in dry CH_2Cl_2 (5 mL per mmol amino acid ester hydrochloride) and stirred under Ar. $N(Et)_3$ (1 mol eq.), the Boc-protected amino acid (1 mol eq.), and PyBOP® (1.5 mol eq.) were next added in this order. The mixture was stirred for 20 hours at room temperature. The solvent was removed *in vacuo* and the title compound was purified via flash chromatography (SiO_2, first with 1:1/ hexane:EtOAc, then switch to EtOAc).

4.1.8. General Procedure for Preparation of Protected Tripeptides (49, 50, 55-57)

The protected dipeptide (1 mol eq.) was dissolved in dry CH_2Cl_2 (3 mL per mmol protected dipeptide) and stirred under Ar. Trifluoroacetic acid (3 mL per mmol protected dipeptide) was added and the mixture was stirred at room temperature for 2 hours. The solvent was removed *in vacuo* and the deprotected dipeptide was placed under high vacuum for 1.5 hours in order to remove trace amounts of solvent. The deprotected dipeptide was re-dissolved in dry CH_2Cl_2 (6 mL per mmol protected dipeptide) under an argon atmosphere. $N(Et)_3$ (2 mol eq.), the Boc-protected amino acid (1 mol eq.), and PyBOP® (1.5 mol eq.) were next added in this order. The mixture was stirred for 20 hours at room temperature. The solvent was removed *in vacuo* and the title compound was purified via flash chromatography (SiO_2, first with 1:1/ hexane:EtOAc, then switch to EtOAc).

4.1.9. General Procedure for Preparation of Protected Dehydro Alanine Containing Tripeptides (42, 51, 52)

The title compounds were prepared by a modification of the procedure of Shin *et al.*[135] The Boc-protected tripeptide (1 mol eq.) was dissolved in dry EtOAc (45 mL per mmol Boc-protected tripeptide) and stirred under Ar. Mesyl chloride (2.1 mol eq.) and DBU (4.3 mol eq.) were next added drop-wise in this order and the mixture was allowed to reflux for 5 hours. After cooling to room temperature, the mixture was washed with H_2O (2 X 50 mL per mmol Boc-protected tripeptide) and brine (1 X 50 mL per mmol Boc-protected tripeptide). The organic layer was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification via flash chromatography (SiO_2, 1:1/hexane:EtOAc) yielded the dehydrated tripeptide.

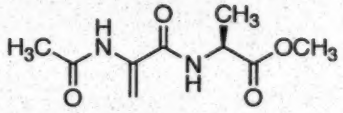
4.1.10. General Procedure for Preparation of Acyl Tripeptides (43, 53, 54, 58, 59)

The title compounds were prepared by a modification of the procedure of Fairlie and coworkers.[159] The Boc-protected dehydrated tripeptide (1 mol eq.) was dissolved in CH_2Cl_2 (8 mL per mmol Boc-protected dehydrated tripeptide). Trifluoroacetic acid (8 mL per mmol Boc-protected dehydrated tripeptide) was added and the mixture was allowed to stir at room temperature for 3 hours. The solvent was removed *in vacuo* and the deprotected tripeptide was placed under high vacuum for 1.5 hours in order to remove trace amounts of solvent. The deprotected tripeptide was re-dissolved in pyridine (16 mL per mmol Boc-protected dehydrated tripeptide). DMAP (1 mol eq.) was added to the

mixture and after cooling to 0 °C, acetic anhydride (5 mol eq.) was added. The mixture was warmed to room temperature and allowed to stir for 2 hours. The solvent was removed *in vacuo* and the crude mixture was re-dissolved in EtOAc (50 mL per mmol tripeptide). The mixture was washed with 10% citric acid (1 X 50 mL per mmol tripeptide), brine (2 X 50 mL per mmol tripeptide), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification via flash chromatography (SiO_2, EtOAc) yielded the desired acylated tripeptide (**54**, **58**, **59**). Compounds **43** and **53** were purified by reverse phase RP-HPLC using a steel walled column (Grace Vydac, 22 x 250 mm, 10 μm). In the method employed a 0.5 mL injection was applied and a gradient of water and acetonitrile (0.1% TFA) was used to purify the acylated tripeptides. Gradient; 30% acetonitrile for 5 min, then climbing to 95% in 10 min returning to 30% in 0.5 min and remaining at this concentration for an additional 5 min (flow rate 10 mL/min., detection at 220 nm). R_t=16.0 min for compound **43**, R_t=15.9 min for compound **53**.

4.1.11. Experimental Data for Compounds

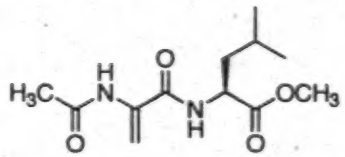
(*S*)-Methyl 2-(2-acetamidoacrylamido)propanoate (24)



Prepared according to the procedure detailed in section 4.1.4 by Dr. Steven Cobb. (White solid, 522 mg, 67%, Rf 0.47 in 100% EtOAc); $[\alpha]_D^{26}$ -13.76° (*c* 0.25, $CHCl_3$); IR ($CDCl_3$ cast) 3343 (br), 3000 (br), 2954, 1740, 1662, 1512 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.99 (br s, 1H, NH), 6.77 (br d, 1H, NH Ala, *J* = 7.1 Hz), 6.46 (d, 1H, C=CH_2, *J* = 1.6

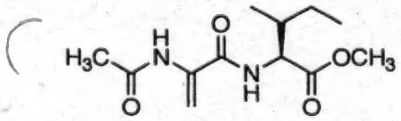
Hz), 5.27 (br s, 1H, C=C$\underline{H}_2$), 4.61 (dq, 1H, α-$\underline{H}$, J = 7.2, 7.1 Hz), 3.75 (s, 3H, OC$\underline{H}_3$), 2.10 (s, 3H, COC$\underline{H}_3$), 1.44 (d, 3H, C$\underline{H}_3$, J = 7.2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0, 169.0, 163.5 (3-$\underline{C}$O), 133.8 ($\underline{C}$=CH$_2$), 101.6 (C=$\underline{C}$H$_2$), 52.7 (O$\underline{C}$H$_3$), 48.6 (α-$\underline{C}$), 24.6 (CO$\underline{C}$H$_3$), 18.1 ($\underline{C}$H$_3$); HRMS (ESMS) Calcd for $C_9H_{14}N_2O_4Na$ 237.0846 found 237.0846 MNa$^+$.

(*S*)-Methyl 2-(2-acetamidoacrylamido)-4-methylpentanoate (25)



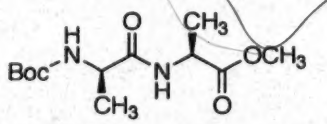
Prepared according to the procedure detailed in section 4.1.4 by Dr. Steven Cobb. (White solid, 575 mg, 58%, Rf 0.40 in 1:2 hexane:EtOAc); $[\alpha]_D^{26}$ +15.53° (*c* 0.77, $CHCl_3$); IR ($CDCl_3$ cast) 3328 (br), 2958, 1746, 1688, 1627, 1510 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.01 (br s, 1H, N$\underline{H}$), 6.71 (d, 1H, N$\underline{H}$ Leu, J = 7.8 Hz), 6.47 (br s, 1H, C=C$\underline{H}_2$), 5.28 (br s, 1H, C=C$\underline{H}_2$), 4.65 (m, 1H, α-$\underline{H}$), 3.74 (s, 3H, OC$\underline{H}_3$), 2.08 (s, 3H, COC$\underline{H}_3$), 1.70-1.55 (m, 3H, C$\underline{H}$, C$\underline{H}_2$ Leu), 0.94-0.91 (m, 6H, 2-C$\underline{H}_3$ Leu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0, 169.0, 163.8 (3-$\underline{C}$O), 133.8 ($\underline{C}$=CH$_2$), 101.6 (C=$\underline{C}$H$_2$), 52.5 (O$\underline{C}$H$_3$), 51.3 (α-$\underline{C}$), 41.4 ($\underline{C}$H$_2$), 24.9 (CO$\underline{C}$H$_3$), 24.6 ($\underline{C}$H), 22.7, 21.8 (2-$\underline{C}$H$_3$); HRMS (ESMS) Calcd for $C_{12}H_{20}N_2O_4Na$ 279.1315 found 279.1314 MNa$^+$.

(2*S*,3*R*)-Methyl 2-(2-acetamidoacrylamido)3-methylpentanoate (26)



Prepared according to the procedure detailed in section 4.1.4 by Dr. Steven Cobb. (Clear oil, 545 mg, 55%, Rf 0.25 in 1:1 hexane:EtOAc); $[\alpha]_D^{26}$ +15.11° (*c* 1.80, $CHCl_3$); IR ($CDCl_3$ cast) 3332 (br), 2965, 2878, 1743, 1657, 1504 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.00 (s, 1H, NH), 6.71 (d, 1H, NH Ile, *J* = 8.1 Hz), 6.44 (br s, 1H, C=CH_2), 5.27 (br s, 1H, C=CH_2), 4.56 (dd, 1H, α-H, *J* = 8.1, 4.9 Hz), 3.71 (s, 3H, OCH_3), 2.06 (s, 3H, COCH_3), 1.90 (m, 1H, CH), 1.43 (m, 1H, CH_2), 1.18 (m, 1H, CH_2), 0.87-0.91 (m, 6H, 2-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.9, 169.0, 163.8 (3-CO), 134.0 (C=CH_2), 101.5 (C=CH_2), 56.9 (α-C), 52.2 (OCH_3), 37.9 (CH), 25.2 (CH_2), 24.6 (COCH_3), 15.4 (CH_3), 11.4 (CH_3); HRMS (ESMS) Calcd for $C_{12}H_{20}N_2O_4Na$ 279.1315 found 279.1315 MNa^+.

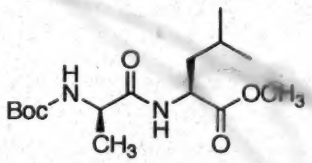
(*S*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)propanoate (31)



Prepared according to the procedure detailed in section 4.1.5 by Ian Armstrong. (Clear oil, 570 mg, 54%, Rf 0.45 in 100% EtOAc); $[\alpha]_D^{26}$ +35.69 ° (*c* 1.48, $CHCl_3$); IR ($CDCl_3$ cast) 3314 (br), 2979, 2936, 1775, 1717, 1663, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.81 (br s, 1H, NH L-Ala), 5.09 (br s, 1H, NH D-Ala), 4.53 (m, 1H, α-H L-Ala), 4.19 (m, 1H, α-H D-Ala), 3.69 (s, 3H, OCH_3), 1.41 (s, 9H, C(CH_3)$_3$), 1.36 (d, 3H, CH_3, *J* = 7.2

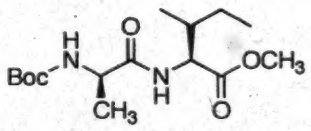
Hz), 1.32 (d, 3H, C<u>H</u>$_3$, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 172.2 (2-<u>C</u>O), 155.5 (<u>C</u>O carbamate), 81.0 (<u>C</u>(CH$_3$)$_3$), 52.4 (O<u>C</u>H$_3$), 50.0 (α-<u>C</u>), 48.0 (α-<u>C</u>), 28.2 (C(<u>C</u>H$_3$)$_3$), 18.2 (2-<u>C</u>H$_3$ Ala); HRMS (ESMS) Calcd for $C_{12}H_{22}N_2O_5Na$ 297.1421 found 279.1420 MNa$^+$.

(*S*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)-4-methylpentanoate (32)



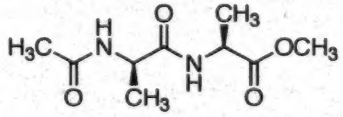
Prepared according to the procedure detailed in section 4.1.5 by Dr. Steven Cobb. (White solid, 902 mg, 75%, Rf 0.63 in 100% EtOAc); $[\alpha]_D^{26}$ +29.12°(*c* 0.98, $CHCl_3$); IR (cast microscope) 3315 (br), 2959, 2873, 17744, 1717, 1664, 1519 (br) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.82 (br s, 1H, N<u>H</u>), 5.22 (d, 1H, N<u>H</u>, J = 6.3 Hz), 4.59 (m, 1H, α-<u>H</u> Leu), 4.18 (m, 1H, α-<u>H</u> Ala), 3.66 (s, 1H, OC<u>H</u>$_3$), 1.47-1.68 (m, 3H, C<u>H</u>$_2$ and C<u>H</u>), 1.39 (s, 9H, C(C<u>H</u>$_3$)$_3$), 1.31 (d, 3H, C<u>H</u>$_3$ Ala, J = 7.2 Hz), 0.87 (d, 6H, 2-C<u>H</u>$_3$ Leu, J = 5.7 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.3, 172.5 (2-<u>C</u>O), 155.5 (<u>C</u>O carbamate), 80.1 (<u>C</u>(CH$_3$)$_3$), 52.2 (O<u>C</u>H$_3$), 50.6 (α-<u>C</u>), 50.0 (α-<u>C</u>), 41.4 (<u>C</u>H$_2$), 28.2 (C(<u>C</u>H$_3$)$_3$), 24.7 (<u>C</u>H), 22.8 (<u>C</u>H$_3$ Leu), 21.8 (<u>C</u>H$_3$ Leu), 18.2 (CH$_3$ Ala); HRMS (ESMS) Calcd for $C_{15}H_{28}N_2O_5Na$ 339.1890 found 339.1891 MNa$^+$.

(2*S*,3*R*)-Methyl 2-((*R*)-2-(*tert*-butoxycarbonylamino)propanamido)-3-methylpentanoate (33)



Prepared according to the procedure detailed in section 4.1.5 by Ian Armstrong. (White solid, 1.109 g, 71%, Rf 0.67 in 100% EtOAc); $[\alpha]_D^{26}$ +52.96° (*c* 0.91, $CHCl_3$); IR ($CDCl_3$ cast) 3313 (br), 2969, 2878, 1743, 1717, 1662, 1521 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.72 (br s, 1H, NH), 4.95 (br s, 1H, NH), 4.53 (dd, 1H, α-H Ile, J = 8.7, 4.8 Hz), 4.17 (m, 1H, α-H Ala), 3.69 (s, 1H, OCH_3), 1.89 (m, 1H, CH Ile), 1.32-1.46 (m, 13H, $C(CH_3)_3$, CH_3 Ala, 1-CH_2), 1.14 (m, 1H, CH_2), 0.86-0.90 (m, 6H, 2-CH_3 Ile); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.4, 172.2 (2-CO), 155.5 (CO carbamate), 80.2 ($C(CH_3)_3$), 56.3 (α-C), 52.0 (OCH_3), 50.1 (α-C), 37.8 (CH Ile), 28.3 ($C(CH_3)_3$), 25.0 (CH_2), 18.1 (CH_3), 15.4 (CH_3), 11.5 (CH_3); HRMS (ESMS) Calcd for $C_{15}H_{28}N_2O_5Na$ 339.1890 found 339.1890 MNa^+.

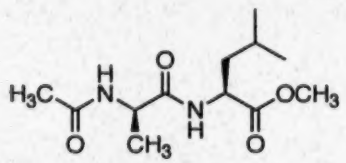
(*S*)-Methyl 2-((*R*)-2-acetamidopropanamido)propanoate (34)



Prepared according to the procedure detailed in section 4.1.6 by Ian Armstrong. (White solid, 151 mg, 45%, Rf 0.45 in 100% EtOAc); $[\alpha]_D^{26}$ +57.69° (*c* 1.65, $CHCl_3$); IR ($CDCl_3$ cast) 3283, 3068, 2984, 2954, 1745, 1652, 1540 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28

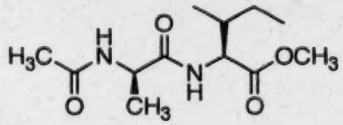
(d, 1H, NH, J = 7.5 Hz), 6.66 (d, 1H, NH, J = 7.5 Hz), 4.55 (m, 1H, α-H), 4.47 (m, 1H, α-H), 3.67 (s, 3H, OCH_3), 1.45 (s, 3H, $COCH_3$), 1.36 (d, 3H, CH_3, J = 7.5 Hz), 1.32 (d, 3H, CH_3, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 172.3, 170.2 (3-CO), 52.3 (OCH_3), 48.6 (CH), 48.1 (CH), 22.9 ($COCH_3$), 18.3 (CH_3), 17.7 (CH_3); HRMS (ESMS) Calcd for $C_9H_{16}N_2O_4Na$ 239.1002 found 239.1002 MNa^+.

(*S*)-Methyl 2-((*R*)-2-acetamidopropanamido)-4-methylpentanoate (35)



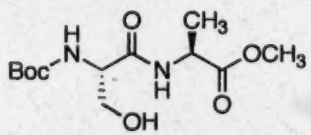
Prepared according to the procedure detailed in section 4.1.6 by Ian Armstrong. (White solid, 469 mg, 70%); $[\alpha]_D^{26}$ +51.06° (*c* 1.13, $CHCl_3$); IR ($CDCl_3$ cast) 3262, 3076, 2955, 1751, 1676, 1557 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.98 (d, 1H, NH, J = 8.4 Hz), 6.41 (d, 1H, NH, J = 6.9 Hz), 4.62-4.48 (m, 2H, 2-α-H), 3.68 (s, 3H, OCH_3), 1.97 (s, 3H, $COCH_3$), 1.71-1.49 (m, 3H, CH_2 Leu, CH Leu), 1.35 (d, 3H, CH_3 Ala, J = 6.7 Hz), 0.92-0.89 (m, 6H, 2-CH_3 Leu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 172.4, 170.2 (3-CO), 52.2 (OCH_3), 50.8 (α-C), 48.6 (α-C), 41.1 (CH_2), 24.8 (CH), 23.1 ($COCH_3$), 22.8 (CH_3 Leu), 21.7 (CH_3 Leu), 18.4 (CH_3 Ala); HRMS (ESMS) Calcd for $C_{12}H_{22}N_2O_4Na$ 281.1472 found 281.1473 MNa^+.

(2*S*,3*R*)-Methyl 2-((*R*)-2-acetamidopropanamido)-3-methylpentanoate (36)



Prepared according to the procedure detailed in section 4.1.6 by Ian Armstrong. (White solid, 197 mg, 26%); $[\alpha]_D^{26}$ +73.00° (*c* 0.85, $CHCl_3$); IR ($CDCl_3$ cast) 3261, 3076, 2972, 1750, 1676, 1635, 1558 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.71 (d, 1H, NH, *J* = 8.7 Hz), 6.11 (d, 1H, NH, *J* = 6.9 Hz), 4.54-4.49 (m, 2H, 2-α-H), 3.70 (s, 3H, OCH_3), 2.00 (s, 3H, $COCH_3$), 1.89 (m, 1H, CH Ile), 1.40 (m, 1H, CH_2), 1.37 (d, 3H, CH_3 Ala, *J* = 7.5 Hz), 1.17 (m, 1H, CH_2), 0.90 (t, 3H, CH_3 Ile, *J* = 7.5 Hz), 0.89 (d, 3H, CH_3 Ile, *J* = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.3, 172.1, 170.1 (3-CO), 56.5 (α-C), 52.1 (OCH_3), 48.7 (α-C), 37.6 (CH Ile), 25.0 (CH_2), 23.1 ($COCH_3$), 18.5 (CH_3 Ala), 15.5 (CH_3 Ile), 11.5 (CH_3 Ile); HRMS (ESMS) Calcd for $C_{12}H_{22}N_2O_4Na$ 281.1472 found 281.1470 MNa^+.

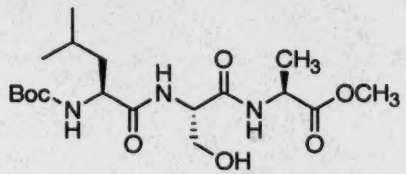
(*S*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)propanoate (38)



Prepared according to the procedure detailed in section 4.1.7. (Yellow oil, 0.91 g, 55%, Rf 0.39 in 100% EtOAc); $[\alpha]_D^{26}$ -7.88° (*c* 0.44, $(CH_3)_2CO$); IR ($(CH_3)_2CO$ cast) 3409 (br), 2980, 1740, 1668 cm^{-1}; ^1H NMR ($(CD_3)_2CO$, 400 MHz) δ 7.61 (br d, 1H, NH, *J* = 6.3

Hz), 6.00 (br s, 1H, NH carbamate), 4.47 (dq, 1H, α-H Ala, J = 7.3, 6.3 Hz), 4.16 (m, 1H, α-H Ser), 3.79 (m, 1H, CH_2 Ser), 3.70 (m, 1H, CH_2 Ser), 3.68 (s, 3H, OCH_3), 2.93 (br s, 1H, OH), 1.41 (s, 9H, $C(CH_3)_3$), 1.36 (d, 3H, CH_3 Ala, J = 7.3 Hz); ^{13}C NMR ($(CD_3)_2CO$, 100 MHz) δ 173.6, 171.1 (2-CO), 156.1 (CO carbamate), 79.3 ($C(CH_3)_3$), 63.2 (CH_2 Ser), 56.8 (α-C Ser), 52.2 (OCH_3), 48.6 (α-C Ala), 28.3 ($C(CH_3)_3$), 17.7 (CH_3 Ala); HRMS (ES) Calcd for $C_{12}H_{22}N_2O_6Na$ 313.1370, found 313.1370 MNa^+.

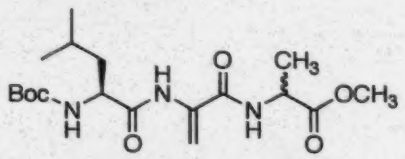
(6*S*,9*S*,12*S*)-Methyl 9-(hydroxymethyl)-6-isobutyl-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecane-12-carboxylate (41)



Compound **38** (0.8 g, 2.9 mmol) was dissolved in dry CH_2Cl_2 (10 mL) under an Ar atmosphere. Trifluoroacetic acid (10 mL) was added and the mixture was allowed to stir at room temperature for 2 hours. The solvent was removed *in vacuo* and the deprotected dipeptide was placed under high vacuum for 1.5 hours in order to remove trace amounts of solvent. The deprotected dipeptide (0.3 g, 1.6 mmol) was dissolved in 10:1/CH_2Cl_2:DMF (11 mL) and stirred under Ar. NMM (0.4 mL, 3.1 mmol), Boc-Leu-OH•H_2O (0.4 g, 1.6 mmol), HOBt (0.4 g, 3.1 mmol), and DIPCDI (0.5 mL, 3.1 mmol) were next added in this order. The mixture was stirred for 20 hours at room temperature. The mixture was washed with H_2O (3 X 20 mL) and brine (1 X 20 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification via flash

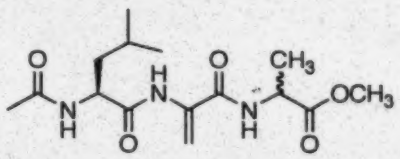
chromatography (SiO_2, EtOAc) yielded an undesired di-coupled product (**40**). The di-coupled product (0.3 g, 0.5 mmol) was dissolved in MeOH (24 mL). Sodium bicarbonate (0.1 g, 1.4 mmol) was added and the mixture was allowed to stir at room temperature for 20 hours. The solvent was removed under reduced pressure and H_2O (20 mL) acidified to pH 2 with 1M HCl was added. The desired crude tripeptide was extracted with EtOAc (2 X 10 mL) and the combined organic layers were washed with brine (2 X 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, first with 1:1/ hexane:EtOAc, then switch to EtOAc, Rf 0.5 in 100% EtOAc) gave the title compound **41** (174 mg, 38%) as a white solid; $[\alpha]_D^{26}$ -47.52° (*c* 0.45, $CHCl_3$); IR ($CHCl_3$ cast) 3302 (br), 3079, 2958, 2872, 1744, 1650 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.25 (br s, 1H, NH Ala), 7.14 (d, 1H, NH Ser, J = 7.5 Hz), 5.04 (m, 1H, NH carbamate), 4.55-4.51 (m, 2H, α-H Ala, α-H Ser), 4.13 (br s, 1H, α-H Leu), 4.03 (dd, 1H, CH_2 Ser, J = 11.5, 3.8 Hz), 3.75 (s, 3H, OCH_3), 3.68 (dd, 1H, CH_2 Ser, J = 11.5, 5.4 Hz), 2.82 (br s, 1H, OH), 1.71-1.61 (m, 2H, CH_2 Leu), 1.50 (m, 1H, CH Leu), 1.43 (s, 9H, $C(CH_3)_3$), 1.43-1.40 (m, 3H, CH_3 Ala), 0.95 (d, 3H, CH_3 Leu, J = 5.3 Hz), 0.94 (d, 3H, CH_3 Leu, J = 5.3 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.2, 173.1, 170.3 (3-CO), 155.7 (CO carbamate), 80.5 ($C(CH_3)_3$), 62.7 (CH_2 Ser), 54.0 (α-C Ser), 52.6 (α-C Leu), 52.2 (OCH_3), 48.4 (α-C Ala), 41.1 (CH_2 Leu), 28.3 ($C(CH_3)_3$), 24.8 (CH Leu), 22.9, 21.8 (2-CH_3 Leu), 17.7 (CH_3 Ala); HRMS (ES) Calcd for $C_{18}H_{33}N_3O_7Na$ 426.2211, found 426.2210 MNa^+.

(*S*)-Methyl 6-isobutyl-2,2-dimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazatridecane-12-carboxylate (42)



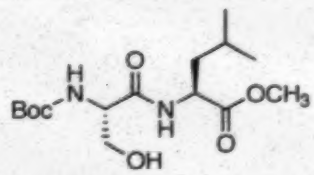
Prepared according to the procedure detailed in section 4.1.9. (Yellow oil, 23 mg, 24%, Rf 0.71 in 100% EtOAc); IR ($CHCl_3$ cast) 3330, 2958, 1742, 1691, 1657, 1631 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.56 (br s, 1H, NH dehydro Ala), 6.72 (br s, 1H, NH Ala), 6.50 (s, 1H, C=CH_2), 5.33 (m, 1H, C=CH_2, both isomers), 4.87 (br s, 1H, NH carbamate), 4.62 (dq, 1H, α-H Ala, J = 7.1, 7.2 Hz), 4.21 (br s, 1H, α-H Leu), 3.79 (s, 3H, OCH_3), 1.71-1.70 (m, 2H, CH_2 Leu), 1.61 (m, 1H, CH Leu), 1.47 (d, 3H, CH_3 Ala, J = 7.2 Hz), 1.45 (s, 9H, C(CH_3)$_3$), 0.96 (d, 3H, CH_3 Leu, J = 6.4 Hz), 0.95 (d, 3H, CH_3 Leu, J = 6.2 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.0, 171.7, 163.2 (3-CO), 155.5 (CO carbamate), 133.7 (C=CH_2), 102.4 (C=CH_2), 80.3 (C(CH_3)$_3$), 54.1 (α-C Leu), 52.7 (OCH_3), 48.6 (α-C Ala), 41.5 (CH_2 Leu), 28.3 (C(CH_3)$_3$), 24.8 (CH Leu), 23.0, 21.8 (2-CH_3 Leu), 18.3 (CH_3 Ala); HRMS (ES) Calcd for $C_{18}H_{31}N_3O_6Na$ 408.2105, found 408.2108 MNa^+.

(*S*)-Methyl 2-(2-((*S*)-2-acetamido-4-methylpentanamido)acrylamido)propanoate (43)



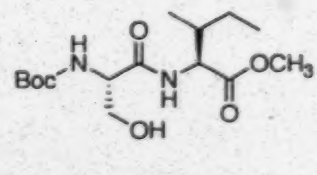
Prepared according to the procedure detailed in section 4.1.10. (Yellow oil, 6 mg, 38%); IR ($CHCl_3$ cast) 3298, 2958, 1743, 1653 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.53 (br s, 1H, NH dehydro Ala), 6.84 (d, 1H, NH Ala, J = 6.9 Hz), 6.23, 6.21 (s, 1H, C=CH_2, both isomers), 6.29 (d, 1H, NH Leu, J = 8.3 Hz), 5.40 (s, 1H, C=CH_2), 4.64 (dq, 1H, α-H Ala, J = 7.2, 6.9 Hz), 4.58 (m, 1H, α-H Leu), 3.80 (s, 3H, OCH_3), 2.08 (s, 3H, $COCH_3$), 1.72-1.70 (m, 2H, CH_2 Leu), 1.57 (m, 1H, CH Leu), 1.49, 1.48 (d, 3H, CH_3 Ala, J = 7.1 Hz, both isomers), 0.97 (d, 3H, CH_3 Leu, J = 4.9 Hz), 0.96 (d, 3H, CH_3 Leu, J = 4.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 171.2, 171.1, 163.2 (4-CO), 133.7 (C=CH_2), 103.5 (C=CH_2), 53.0 (OCH_3), 53.0 (α-C Leu), 48.7, 48.1 (α-C Ala, both isomers), 41.4, 40.9 (CH_2 Leu, both isomers), 25.0 (CH Leu), 23.2 ($COCH_3$), 23.1, 22.2 (2-CH_3 Leu), 18.5 (CH_3 Ala); HRMS (ES) Calcd for $C_{15}H_{25}N_3O_5Na$ 350.1686, found 350.1689 MNa^+.

(*S*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)-4-methylpentanoate (45)



Prepared according to the procedure detailed in section 4.1.7. (Yellow oil, 2.38 g, 65%, Rf 0.51 in 100% EtOAc); $[\alpha]_D^{26}$ -37.46° (*c* 2.34, $CHCl_3$); IR ($CDCl_3$ cast) 3315 (br), 2959, 2873, 1721, 1665 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.90 (m, 1H, NH), 5.55 (m, 1H, NH carbamate), 4.59 (m, 1H, α-H Leu), 4.18 (m, 1H, α-H Ser), 4.07 (m, 1H, CH_2 Ser), 3.74 (s, 3H, OCH_3), 3.64 (dd, 1H, CH_2 Ser, J = 11.4, 5.4 Hz), 2.75 (br s, 1H, OH), 1.68-1.55 (m, 3H, CH_2, CH Leu), 1.46 (s, 9H, $C(CH_3)_3$), 0.94 (d, 3H, CH_3 Leu, J = 5.9 Hz), 0.92 (d, 3H, CH_3 Leu, J = 5.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 171.4 (2-CO), 156.0 (CO carbamate), 80.3 ($C(CH_3)_3$), 62.8 (CH_2 Ser), 54.5 (α-C Ser), 52.3 (OCH_3), 50.8 (α-C Leu), 40.8 (CH_2 Leu), 28.2 ($C(CH_3)_3$), 24.7 (CH Leu), 22.7, 21.5 (2-CH_3 Leu); HRMS (ES) Calcd for $C_{15}H_{28}N_2O_6Na$ 355.1840, found 355.1840 MNa^+.

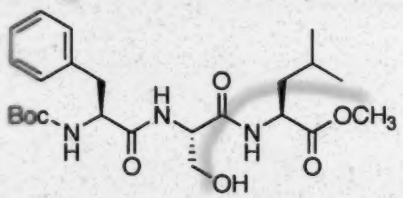
(2*S*,3*R*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxypropanamido)-3-methylpentanoate (46)



Prepared according to the procedure detailed in section 4.1.7. (Yellow oil, 2.28 g, 62%, Rf 0.46 in 100% EtOAc); $[\alpha]_D^{26}$ -34.73° (*c* 2.24, $CHCl_3$); IR ($CDCl_3$ cast) 3321 (br), 2968,

2935, 2879, 1742, 1721, 1665 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.14 (br d, 1H, NH, *J* = 5.4 Hz), 5.62 (d, 1H, NH carbamate, *J* = 7.4 Hz), 4.53 (dd, 1H, α-H Ile, *J* = 8.6, 5.4 Hz), 4.18 (m, 1H, α-H Ser), 4.06 (m, 1H, CH_2 Ser), 3.73 (s, 3H, OCH_3), 3.64 (dd, 1H, CH_2 Ser, *J* = 10.8, 3.7 Hz), 3.24 (br s, 1H, OH), 1.91 (m, 1H, CH, Ile), 1.44 (s, 9H, $C(CH_3)_3$), 1.39 (m, 1H, CH_2 Ile), 1.17 (m, 1H, CH_2 Ile), 0.91-0.89 (m, 6H, 2-CH_3 Ile); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.0, 171.5 (2-CO), 156.1 (CO carbamate), 80.3 ($C(CH_3)_3$), 62.6 (CH_2 Ser), 56.6 (α-C Ile), 54.4 (α-C Ser), 52.1 (OCH_3), 37.3 (CH Ile), 28.1 ($C(CH_3)_3$), 24.8 (CH_2 Ile), 15.4, 11.4 (2-CH_3 Ile); HRMS (ES) Calcd for $C_{15}H_{28}N_2O_6Na$ 355.1840, found 355.1840 MNa^+.

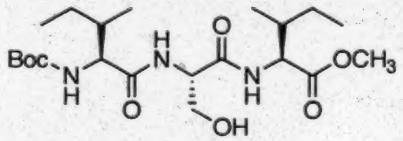
(6*S*,9*S*,12*S*)-Methyl 6-benzyl-9-(hydroxymethyl)-2,2,14-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (49)



Prepared according to the procedure detailed in section 4.1.8. (White solid, 0.30 g, 43%, Rf 0.41 in 100% EtOAc); $[\alpha]_D^{26}$ -21.27° (*c* 1.38, $CHCl_3$); IR ($CDCl_3$ cast) 3306 (br), 3065, 2960, 2873, 1742, 1692, 1643 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.30-7.14 (m, 7H, 5-ArH, NH Ser, NH Leu), 5.16 (br s, 1H, NH carbamate), 4.54-4.52 (m, 2H, α-H Leu, α-H Ser), 4.41 (m, 1H, α-H Phe), 3.95 (dd, 1H, CH_2 Ser, *J* = 11.4, 4.3 Hz), 3.72 (s, 3H, OCH_3), 3.63 (dd, 1H, CH_2 Ser, *J* = 11.4, 5.6 Hz), 3.31 (br s, 1H, OH), 3.12 (dd, 1H, CH_2 Phe, *J* = 13.9, 5.8 Hz), 3.06 (m, 1H, CH_2 Phe), 1.68-1.58 (m, 3H, CH_2, CH Leu), 1.37 (s,

9H, C(C<u>H</u>$_3$)$_3$), 0.94 (d, 3H, C<u>H</u>$_3$ Leu, J = 6.0 Hz), 0.92 (d, 3H, C<u>H</u>$_3$ Leu, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.3, 172.2, 170.3 (3-<u>C</u>O), 155.5 (<u>C</u>O carbamate), 136.1 (Ar–<u>C</u> quaternary), 129.1, 128.6, 126.9 (3-Ar-<u>C</u>), 80.4 (<u>C</u>(CH$_3$)$_3$), 62.6 (<u>C</u>H$_2$ Ser), 55.7 (α-<u>C</u> Phe), 54.2 (α-<u>C</u> Ser), 52.4 (O<u>C</u>H$_3$), 51.1 (α-<u>C</u> Leu), 40.4 (<u>C</u>H$_2$ Leu), 38.0 (<u>C</u>H$_2$ Phe), 28.1 (C(<u>C</u>H$_3$)$_3$), 24.7 (<u>C</u>H Leu), 22.7, 21.5 (2-<u>C</u>H$_3$ Leu); HRMS (ES) Calcd for $C_{24}H_{37}N_3O_7Na$ 502.2524, found 502.2526 MNa^+.

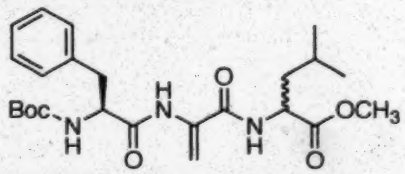
(6*S*,9*S*,12*S*,13*R*)-Methyl 6-*sec*-butyl-9-(hydroxymethyl)-2,2,13-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (50)



Prepared according to the procedure detailed in section 4.1.8. (White solid, 0.25 g, 24%, Rf 0.34 in 100% EtOAc); $[\alpha]_D^{26}$ -24.41° (*c* 1.19, $CHCl_3$); IR ($CHCl_3$ cast) 3281 (br), 3088, 2965, 2934, 2878, 1741, 1691, 1645 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.18 (d, 1H, N<u>H</u> Ser, J = 8.3 Hz), 7.12 (d, 1H, N<u>H</u> Ile, J = 6.8 Hz), 5.16 (d, 1H, N<u>H</u> carbamate, J = 6.2 Hz), 4.55-4.48 (m, 2H, α-<u>H</u> Ile, α-<u>H</u> Ser), 4.01-3.98 (m, 2H, α-<u>H</u> Ile, C<u>H</u>$_2$ Ser), 3.72 (s, 3H, O<u>C</u>H$_3$), 3.64 (dd, 1H, C<u>H</u>$_2$ Ser, J = 11.5, 5.5 Hz), 2.89 (br s, 1H, O<u>H</u>), 1.88-1.86 (m, 2H, 2-C<u>H</u> Ile), 1.46-1.43 (m, 2H, C<u>H</u>$_2$ Ile), 1.41 (s, 9H, C(C<u>H</u>$_3$)$_3$), 1.21-1.08 (m, 2H, C<u>H</u>$_2$ Ile), 0.92-0.86 (m, 12H, 4-C<u>H</u>$_3$ Ile); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.7, 171.9, 170.5 (3-<u>C</u>O), 155.8 (<u>C</u>O carbamate), 80.2 (<u>C</u>(CH$_3$)$_3$), 62.5 (<u>C</u>H$_2$ Ser), 59.3, 56.9 (2-α-<u>C</u> Ile), 54.0 (α-<u>C</u> Ser), 52.1 (O<u>C</u>H$_3$), 37.3, 37.0 (2-<u>C</u>H Ile), 28.1 (C(<u>C</u>H$_3$)$_3$), 25.0, 24.6 (2-<u>C</u>H$_2$

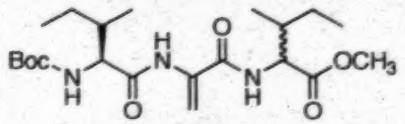
Ile), 15.5, 15.4, 11.4, 11.3 (4-$\underline{C}H_3$ Ile); HRMS (ES) Calcd for $C_{21}H_{39}N_3O_7Na$ 468.2680, found 468.2680 MNa^+.

(*S*)-Methyl 6-benzyl-2,2,14-trimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (51)



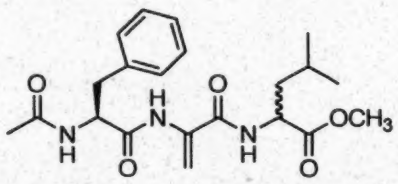
Prepared according to the procedure detailed in section 4.1.9. (Yellow oil, 12 mg, 12%, Rf 0.78 in 100% EtOAc); IR ($CDCl_3$ cast) 3330, 2958, 2871, 1734, 1685, 1653, 1634 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.42, 8.41 (br s, 1H, N$\underline{H}$ dehydro Ala, both isomers), 7.32-7.18 (m, 5H, Ar$\underline{H}$), 6.52-6.50 (m, 2H, N$\underline{H}$ Leu, C=C$\underline{H}_2$), 5.34, 5.32 (s, 1H, C=C$\underline{H}_2$, both isomers), 4.94 (br s, 1H, N$\underline{H}$ carbamate), 4.65 (m, 1H, α-$\underline{H}$ Leu), 4.45 (br s, 1H, α-$\underline{H}$ Phe), 3.76 (s, 3H, OC$\underline{H}_3$), 3.18, 3.14 (m, 1H, C$\underline{H}_2$ Phe, both isomers), 3.07 (m, 1H, C$\underline{H}_2$ Phe), 1.72-1.58 (m, 3H, C$\underline{H}_2$, C$\underline{H}$ Leu), 1.40 (s, 9H, C(C$\underline{H}_3$)$_3$), 0.96 (d, 3H, C$\underline{H}_3$ Leu, J = 5.9 Hz), 0.94 (d, 3H, C$\underline{H}_3$ Leu, J = 5.9 Hz); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 173.0, 170.2, 163.2 (4-$\underline{C}$O), 155.3 ($\underline{C}$O carbamate), 136.1, 136.1 (Ar-$\underline{C}$ quaternary, both isomers), 133.5, 133.5 ($\underline{C}$=CH$_2$, both isomers), 129.1, 128.6, 126.9 (3-Ar-$\underline{C}$), 102.4 (C=$\underline{C}$H$_2$), 80.4 ($\underline{C}$(CH$_3$)$_3$), 56.4 (α-$\underline{C}$ Phe), 52.4 (O$\underline{C}$H$_3$), 51.2 (α-$\underline{C}$ Leu), 41.5, 41.4 ($\underline{C}$H$_2$ Leu, both isomers), 38.2 ($\underline{C}$H$_2$ Phe), 28.1 (C($\underline{C}$H$_3$)$_3$), 24.8 ($\underline{C}$H Leu), 22.7, 22.6, 21.9, 21.8 (2-$\underline{C}$H$_3$ Leu, both isomers); HRMS (ES) Calcd for $C_{24}H_{35}N_3O_6Na$ 484.2418, found 484.2420 MNa^+.

(6*S*,12*S*,13*R*)-Methyl 6-*sec*-butyl-2,2,13-trimethyl-9-methylene-4,7,10-trioxo-3-oxa-5,8,11-triazapentadecane-12-carboxylate (52)



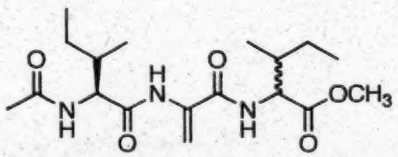
Prepared according to the procedure detailed in section 4.1.9. (Yellow oil, 31 mg, 32%, Rf 0.77 in 100% EtOAc); IR ($CHCl_3$ cast) 3323, 2966, 2935, 2878, 1736, 1690, 1662, 1633 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.45 (br s, 1H, NH dehydro Ala), 6.71 (d, 1H, NH Ile, J = 8.1 Hz), 6.51 (d, 1H, C=CH_2, J = 1.7 Hz), 5.32 (m, 1H, C=CH_2, both isomers), 5.04 (d, 1H, NH carbamate, J = 6.4 Hz), 4.60 (dd, 1H, α-H Ile, J = 8.3, 5.2 Hz), 4.11 (m, 1H, α-H Ile), 3.77 (s, 3H, OCH_3), 1.94-1.91 (m, 2H, 2-CH Ile), 1.48-1.46 (m, 2H, CH_2 Ile), 1.44 (s, 9H, C(CH_3)$_3$), 1.28-1.09 (m, 2H, CH_2 Ile), 0.96-0.91 (m, 12H, 4-CH_3 Ile); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.9, 170.6, 163.4 (3-CO), 155.6 (CO carbamate), 133.5 (C=CH_2), 102.0 (C=CH_2), 80.0 (C(CH_3)$_3$), 60.0, 56.9 (2-α-C Ile), 52.2 (OCH_3), 37.8, 37.4 (2-CH Ile), 28.1 (C(CH_3)$_3$), 25.0, 24.5 (2-CH_2 Ile), 15.5, 15.3, 11.4, 11.3 (4-CH_3 Ile); HRMS (ES) Calcd for $C_{21}H_{37}N_3O_6Na$ 450.2575, found 450.2576 MNa^+.

(*S*)-Methyl 2-(2-(2-acetamido-3-phenylpropanamido)acrylamido)-4-methylpentanoate (53)



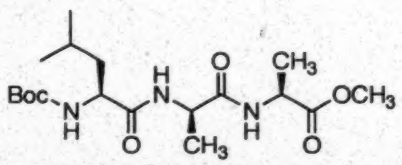
Prepared according to the procedure detailed in section 4.1.10. (Yellow oil, 9 mg, 33%); IR ($CDCl_3$ cast) 3305, 3030, 2958, 2871, 1742, 1656 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.29, 8.27 (s, 1H, NH dehydro Ala, both isomers), 7.31-7.14 (m, 5H, ArH), 6.62, 6.55 (m, 1H, NH Leu, both isomers) 6.41, 6.40 (d, 1H, C=CH_2, J = 1.6 Hz, both isomers), 6.34, 6.32 (m, 1H, NH Phe, both isomers), 5.38 (s, 1H, C=CH_2), 4.77 (m, 1H, α-H Leu), 4.64 (m, 1H, α-H Phe), 3.77, 3.76 (s, 3H, OCH_3, both isomers), 3.11-3.09 (m, 2H, CH_2 Phe), 2.02 (s, 3H, $COCH_3$), 1.72-1.58 (m, 3H, CH_2, CH Leu), 0.96-0.94 (m, 6H, 2-CH_3 Leu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 173.1 (CO, both isomers), 170.9, 169.9, 163.2 (3-CO), 135.8, 135.7 (Ar–C quaternary, both isomers), 133.6 (C=CH_2), 129.2, 128.8, 127.3 (3-Ar-C), 103.6 (C=CH_2), 55.3 (α-C Phe), 52.6 (OCH_3), 51.4 (α-C Leu), 41.6, 41.5 (CH_2 Leu, both isomers), 38.2 (CH_2 Phe), 24.9 (CH Leu), 23.0 ($COCH_3$), 22.8, 22.0 (2-CH_3 Leu); HRMS (ES) Calcd for $C_{21}H_{29}N_3O_5Na$ 426.1999, found 426.1999 MNa^+.

(2*S*,3*R*)-Methyl 2-(2-((2S,3S)-2-acetamido-3-methylpentanamido)acrylamido)-3-methylpentanoate (54)



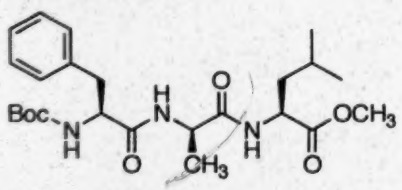
Prepared according to the procedure detailed in section 4.1.10. (White solid, 24 mg, 49%, Rf 0.49 in 100% EtOAc); IR ($CHCl_3$ cast) 3294, 2965, 2936, 2878, 1734, 1685, 1655 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.37 (br s, 1H, NH dehydro Ala), 6.73 (d, 1H, NH C-terminal Ile, J = 7.9 Hz), 6.46 (s, 1H, C=CH_2, both isomers), 6.17 (d, 1H, NH N-terminal Ile, J = 7.7 Hz), 5.35 (m, 1H, C=CH_2, both isomers), 4.60 (dd, 1H, α-H C-terminal Ile, J = 7.9, 5.1 Hz), 4.44 (dd, 1H, α-H N-terminal Ile , J = 7.7, 6.3 Hz), 3.77 (s, 3H, OCH_3), 2.03 (s, 1H, $COCH_3$), 1.98-1.86 (m, 2H, 2-CH Ile), 1.57-1.43 (m, 2H, CH_2 Ile), 1.41 (s, 9H, $C(CH_3)_3$), 1.28-1.09 (m, 2H, CH_2 Ile), 0.96-0.90 (m, 12H, 4-CH_3 Ile); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 172.0, 170.4, 170.0, 163.4 (4-CO), 133.7 (C=CH_2), 102.6 (C=CH_2), 58.5, 57.0 (2-α-C Ile), 52.3 (OCH_3), 38.0, 37.6 (2-CH Ile), 25.3, 24.9 (2-CH_2 Ile), 23.2 ($COCH_3$), 15.4, 15.4, 11.5, 11.4 (4-CH_3 Ile); HRMS (EI) Calcd for $C_{18}H_{31}N_3O_5Na$ 392.2156, found 392.2154 MNa^+.

(6*S*,9*R*,12*S*)-Methyl 6-isobutyl-2,2,9,12-tetramethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (55)



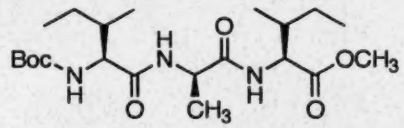
Prepared according to the procedure detailed in section 4.1.8 by Ian Armstrong. (White solid, 170 mg, 85% purity as determined by ^{1}H NMR, Rf 0.46 in 100% EtOAc); IR ($CDCl_3$ cast) 3299 (br), 2959, 2873, 1746, 1652, 1521 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.93 (br s, 1H, NH), 6.57 (d, 1H, NH, *J* = 7.6 Hz), 4.89 (br s, 1H, NH), 4.58-4.48 (m, 2H, 2-α-H Ala), 4.03 (m, 1H, α-H Leu), 3.71 (s, 3H, OCH_3), 1.70-1.62 (m, 3H, CH_2, CH), 1.50-1.35 (m, 15H, $C(CH_3)_3$, 2-CH_3 Ala), 0.94-0.91 (m, 6H, 2-CH_3 Leu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 172.6, 171.6 (3-CO), 156.0 (CO carbamate), 80.3 ($C(CH_3)_3$), 53.6 (α-C Leu), 52.4 (OCH_3), 48.5 (α-C Ala), 48.1 (α-C Ala), 40.9 (CH_2), 28.2 ($C(CH_3)_3$), 24.8 (CH Leu), 22.9 (CH_3 Leu), 21.9 (CH_3 Leu), 17.9 (CH_3 Ala), 17.7 (CH_3 Ala); HRMS (ESMS) Calcd for $C_{18}H_{33}N_3O_6Na$ 410.2262 found 410.2262 MNa^+.

(6*S*,9*R*,12*S*)-Methyl 6-benzyl-12-isobutyl-2,2,9-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (56)



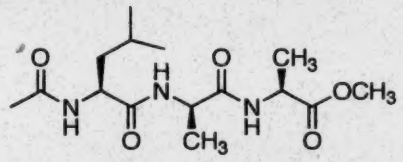
Prepared according to the procedure detailed in section 4.1.8. (White solid, 2.98 g, 90% purity as determined by ^{1}H NMR, Rf 0.57 in 100% EtOAc); IR ($CDCl_3$ cast) 3296 (br), 3064, 2958, 1745, 1645, 1521 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.32-7.18 (m, 5H, ArH), 6.90 (d, 1H, NH, *J* = 6.9 Hz), 6.35 (d, 1H, NH, *J* = 6.9 Hz), 5.14 (d, 1H, NH, *J* = 6.9 Hz), 4.67-4.45 (m, 2H, α-H Ala, α-H Leu), 4.26 (m, 1H, α-H Phe), 3.69 (s, 3H, OCH_3), 3.12-3.01 (m, 2H, CH_2 Phe), 1.69-1.53 (m, 3H, CH_2, CH Leu), 1.39 (s, 9H, $C(CH_3)_3$), 1.21 (d, 3H, CH_3 Ala, *J* = 7.2 Hz), 0.96-0.91 (m, 6H, 2-CH_3 Leu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 171.8, 171.1 (3-CO), 155.3 (CO carbamate), 136.6 (Ar–C quaternary), 129.2, 128.6, 126.9 (3-Ar-C), 80.1 ($C(CH_3)_3$), 56.3 (α-C Phe), 52.2 (OCH_3), 50.7 (α-C Leu), 48.6 (α-C Ala), 41.1 (CH_2 Leu), 38.5 (CH_2 Phe), 28.2 ($C(CH_3)_3$), 24.8 (CH Leu), 22.7, 21.8, (2-CH_3 Leu), 17.9 (CH_3 Ala); ^{13}C NMR ($CDCl_3$, MHz) δ; HRMS (ESMS) Calcd for $C_{24}H_{37}N_3O_6Na$ 486.2575 found 486.2572 MNa^+.

(6*S*,9*R*,12*S*)-Methyl 6,12-di-*sec*-butyl-2,2,9-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (57)



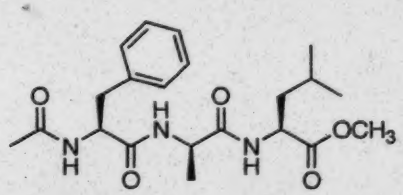
Prepared according to the procedure detailed in section 4.1.8 by Ian Armstrong. (White solid, 346 mg, 37%, Rf 0.71 in 100% EtOAc); $[\alpha]_D^{26}$ +28.04° (*c* 1.08, $CHCl_3$); IR (DCM, cast) 3299, 2967, 2934, 2877, 1641, 1519 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.94 (d, 1H, NH, *J* = 8.8 Hz), 6.66 (d, 1H, NH, *J* = 7.3 Hz), 5.13 (br s, 1H, NH), 4.59 (m, 1H, α-H Ala), 4.52 (dd, 1H, α-H Ile, *J* = 8.8, 5.6 Hz), 3.96 (m, 1H, α-H Ile), 3.69 (s, 3H, OCH_3), 1.89-1.87 (m, 2H, 2-CH Ile), 1.52-1.43 (m, 2H, CH_2), 1.41 (s, 9H, $C(CH_3)_3$), 1.37 (d, 3H, CH_3, *J* = 6.8 Hz), 1.20-1.03 (m, 2H, CH_2), 0.91-0.86 (m, 12H, 4-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.1, 171.9, 171.6, (3-CO), 155.5 (CO carbamate), 79.8 ($C(CH_3)_3$), 59.4, 56.5 (2-α-C Ile), 52.1 (OCH_3), 48.7 (α-C Ala), 37.7, 37.4 (2-CH Ile), 28.3 ($C(CH_3)_3$), 25.1, 24.7 (2-CH_2 Ile), 18.5 (CH_3 Ala), 15.5, 15.5 (2-CH_3 Ile), 11.5, 11.5 (2-CH_3 Ile); HRMS (ESMS) Calcd for $C_{21}H_{39}N_3O_6Na$ 452.2731 found 452.2733 MNa^+.

(*S*)-Methyl 2-((*R*)-2-((*S*)-2-acetamido-4-methylpentanamido)propanamido)propanoate (58)



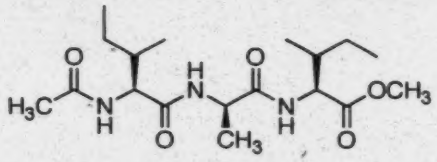
Prepared according to the procedure detailed in section 4.1.10. (White solid, 57 mg, 11%); $[\alpha]_D^{26}$ -11.17° (*c* 0.85, $CHCl_3$); IR ($CHCl_3$ cast) 3286, 3065, 2957, 2873, 1742, 1655 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.19 (d, 1H, NH, *J* = 7.6 Hz), 6.86 (d, 1H, NH, *J* = 8.1 Hz), 6.27 (d, 1H, NH, *J* = 7.3 Hz), 4.54 (dq, 1H, α-H Ala, *J* = 7.3, 7.3 Hz), 4.53 (dq, 1H, α-H Ala, *J* = 7.6, 7.3 Hz), 4.34 (m, 1H, α-H Leu), 3.73 (s, 3H, OCH_3), 1.99 (s, 3H, $COCH_3$), 1.72-1.61 (m, 2H, CH_2 Leu), 1.54 (m, 1H, CH Leu), 1.40 (d, 3H, CH_3 Ala, *J* = 7.3 Hz), 1.39 (d, 3H, CH_3 Ala, *J* = 7.3 Hz), 0.96 (d, 3H, CH_3 Leu, *J* = 6.3 Hz), 0.93 (d, 3H, CH_3 Leu, *J* = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 173.6, 172.4, 171.6, 170.9 (4-CO), 52.5 (OCH_3), 52.4 (α-C Leu), 48.8, 48.1 (2-α-C Ala), 40.6 (CH_2 Leu), 24.8 ($COCH_3$), 23.0 (CH Leu), 22.8, 22.3 (2-CH_3 Leu), 17.8, 17.7 (CH_3 Ala); HRMS (ES) Calcd for $C_{15}H_{27}N_3O_5Na$ 352.1845, found 352.1843 MNa^+.

(*S*)-Methyl 2-((*R*)-2-((*S*)-2-acetamido-3-phenylpropanamido)propanamido)-4-methylpentanoate (59)



Prepared according to the procedure detailed in section 4.1.10. (White solid, 299 mg, 69%, Rf 0.38 in 100% EtOAc); $[\alpha]_D^{26}$ 36.49° (*c* 1.06, $CHCl_3$); IR ($CDCl_3$ cast) 3289, 3065, 2958, 2872, 1741, 1650 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.32-7.19 (m, 5H, ArH), 7.00 (d, 1H, NH , *J* = 8.4 Hz), 6.34 (d, 1H, NH, *J* = 6.9 Hz), 6.28 (d, 1H, NH, *J* = 7.8 Hz), 4.60-4.44 (m, 3H, α-H Phe, α-H Ala, α-H Leu), 3.70 (s, 3H, OCH_3), 3.10-3.00 (m, 2H, CH_2 Phe), 1.98 (s, 3H, $COCH_3$), 1.62-1.60 (m, 3H, CH_2, CH Leu), 1.16 (d, 3H, CH_3 Ala, *J* = 7.1 Hz), 0.92 (d, 3H, CH_3 Leu, *J* = 6.4 Hz), 0.91 (d, 3H, CH_3 Leu, *J* = 6.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.8, 171.9, 171.1, 170.4 (4-CO), 136.5 (Ar–C quaternary), 129.3, 128.6, 127.0 (3-Ar-C), 55.3 (α-C Phe), 52.2 (OCH_3), 50.6 (α-C Leu), 48.6 (α-C Ala), 41.1 (CH_2 Leu), 38.5 (CH_2 Phe), 24.8 (CH Leu), 23.0 ($COCH_3$), 22.9, 21.7, (2-CH_3 Leu), 18.1 (CH_3 Ala); HRMS (ES) Calcd for $C_{21}H_{31}N_3O_5Na$ 428.2159, found 428.2156 MNa^+.

(2*S*,3*R*)-Methyl 2-((*R*)-2-((2*S*,3*S*)-2-acetamido-3-methylpentanamido)propanamido)-3-methylpentanoate (60)



Prepared according to the procedure detailed in section 4.1.6 by Ian Armstrong. (White solid, 86 mg, 27%); $[\alpha]_D^{26}$ +12.93° (*c* 0.28, $CHCl_3$); IR (CH_2Cl_2, cast) 3279, 3068, 2963, 2932, 2875, 2797, 1732, 1662, 1629, 1548 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.22 (d, 1H, NH, *J* = 7.2 Hz), 6.83 (d, 1H, NH, *J* = 7.5 Hz), 6.44 (d, 1H, NH, *J* = 8.7 Hz), 4.65 (m, 1H, α-H Ala), 4.57 (dd, 1H, α-H Ile, *J* = 8.7, 4.8 Hz), 4.24 (apparent t, 1H, α-H Ile), 3.70 (s, 3H, OCH_3), 2.00 (s, 3H, $COCH_3$), 1.93-1.76 (m, 2H, 2-CH Ile), 1.52-1.32 (m, 2H, CH_2 Ile), 1.38 (d, 3H, CH_3 Ala, *J* = 7.2 Hz), 1.21-1.05 (m, 2H, CH_2 Ile), 0.91-0.86 (m, 12H, 4-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.7, 171.8, 171.3, 170.4 (4-CO), 57.9 (α-C Ile), 56.4 (α-C Ile), 52.2 (OCH_3), 48.9 (α-C Ala), 38.0, 37.4 (2-CH Ile), 25.2, 25.1 (2-CH_2 Ile), 23.2 ($COCH_3$), 18.8 (CH_3 Ala), 15.5, 15.3 (2-CH_3 Ile), 11.5, 11.4 (2-CH_3 Ile); HRMS (ESMS) Calcd for $C_{18}H_{33}N_3O_5Na$ 394.2312 found 394.2309 MNa^+.

4.2. Lacticin 3147 LtnJ

4.2.1. Bacterial Strains, Plasmids, and Culture Media

The plasmids and producer strains utilized in the study of lacticin 3147 LtnJ are listed in Table 5. Plasmids pPC1 and pPC2 were obtained from P. Cotter (University College Cork, Ireland). Bacterial strains were obtained from the following suppliers: *E. coli* DH5α, Invitrogen; *E. coli* BL21(DE3), and *E. coli* Origami™B(DE3), Novagen; *E. coli* TB1, *E. coli* K12 CAG629, *E. coli* K12 UT5600, *E. coli* K12 ER2507, and *E. coli* K12 ER2508, New England Biolabs Inc.. Bacterial strains, stored in LB broth (Luria-Bertani) supplemented with 20% (v/v) glycerol, were maintained as frozen stock cultures at -80 °C. *E. coli* was grown in LB or TB broth at 37 °C unless otherwise stated. LB (Luria-Bertani), Tryptone, Yeast Extract, and Agar were provided by Difco (Aldrich) and prepared according to manufacturer's directions. Solid media was prepared by addition of granulated agar 1.5% (w/v) to the broth media. Terrific Broth (TB) was prepared as follows: 1.2 g Tryptone, 2.4 g Yeast Extract, and 0.4 mL glycerol in 90 mL H_2O and 10 mL 10x TB salts. The 10x TB salts are sterilized separately and contains 170 mM KH_2PO_4 and 720 mM K_2HPO_4. When appropriate, antibiotics were added to the *E. coli* growth media at the following selective concentrations: ampicillin (100 μg/mL), kanamycin (15 μg/mL), and tetracycline (12.5 μg/mL).

Table 5. Bacterial strains and plasmids used in LtnJ project.

Strain or plasmid	Description[a, b]	Reference or source
Strains		
L. lactis subsp. *lactis* DPC3147	Lacticin 3147 producer strain	[109]
L. lactis subsp. *lactis* MG1363	Host strain for lacticin 3147 overexpression	[160]
E. coli DH5α	F 80d*lacZ*M15 (*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1	[161]
E. coli BL21(DE3)	F *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3); bacteriophage DE3 lysogen carrying the T7 RNA polymerase gene controlled by the lacUV5 promoter, general purpose expression host	[162]
E. coli Origami™ B(DE3)	F *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* lacY1 ahpC gor522::Tn10(Tc^R) trx::kan^R, tet^R (DE3)	[163]
E. coli K12 TB1	F *ara* Δ*(lac-proAB) [Φ80dlac* Δ*(lacZ)M15] rpsL*(Str^R) *thi hsdR*	[164], [165]
E. coli K12 CAG629	F *lacZ*(am) *pho*(am) *lon supC*(ts) *trp*(am) *rpsL rpoH*(am)*165 zhg::Tn*10 *mal*(am)	[166]
E. coli K12 UT5600	F *ara-14 leuB6 secA6 lacY1 proC14 tsx-67* Δ*(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*	[167], [168], [169]
E. coli K12 ER2507	F *ara-14 leuB6 fhuA2* Δ*(argF-lac)U169 lacY1 glnV44 galK2 rpsL20 xyl-5 mtl-5* Δ*(malB) zjc::Tn*5(Kan^s) Δ$(mcrC\text{-}mrr)_{NEB10}$	[170]
E. coli K12 ER2508	F *ara-14 leuB6 fhuA2* Δ*(argF-lac)U169 lacY1 lon::miniTn*10(Tet^s) *glnV44 galK2 rpsL20*(Str^s) *xyl-5 mtl-5* Δ*(malB) zjc::Tn*5(Kan^s) Δ$(mcrC\text{-}mrr)_{NEB10}$	[171], [172]
Plasmids		
pMRC01	60.2-kb lactococcal plasmid containing *ltnEFIRA1A2M1TM2J*	[119]
pMRC01 Δ*ltnJ*	59.1-kb derivative of pMRC01 with *ltnJ* deletion	[117]
pOM02	pCI372-derivative containing *ltnEFIRA1A2M1TM2J*	[173]
pET-32 Xa/LIC	amp^R, 5926-bp, T7*lac*, f1 ori, His tag (N,C), S tag (N), Trx tag (N), protease T,X, pET expression vector	[174]
pPC1	pET-32 Xa/LIC containing *ltnJ* fragment, His	This study

	tag (N)	
pPC2	pET-32 Xa/LIC containing *ltnJ* fragment, His tag (N, C)	This study
pET15b	Amp^R, 5708-bp, T7*lac*, His Tag (N), pET expression vector	[175]
pET11-a	Amp^R, 5677-bp, T7*lac*, pET expression vector	[176]
pMAL™ c2X	Amp^r, 6648-bp, *lacI*, *lacZα* and *malE* expression vector	[177]
pLS1A	pET11-a containing *ltnJ* fragment	This study
pLS1B	pET11-a containing *ltnJ* fragment, His tag (C)	This study
pLS2	pET15b containing *ltnJ* fragment, His tag (N)	This study
pLS3	pMAL™ c2X containing *ltnJ* fragment	This study

[a] N = N-terminus, C = C-terminus
[b] Protease cleavage sites: T = thrombin, X = Factor Xa

4.2.2. DNA Manipulations and Transformations

Cloning and DNA manipulations were performed as described by Sambrook *et al.*[178] Restriction enzymes *Nde*I, *Bam*HI, and *Eco*RI were obtained from Invitrogen and *Xmn*I from New England Biolabs Inc. Restriction enzyme digestions were performed according to the manufacturer's instructions. T4 DNA ligase and Platinum *Taq* Hi Fidelity polymerase were purchased from Invitrogen. *E. coli* transformations were performed according to Short Protocols in Molecular Biology[179] or using TransformAid Bacterial Transformation System from Fermentas. QIAquick™ Gel Extraction Kit, QIAquick™ PCR Purification Kit, and QIAprep™ Spin Miniprep Kit were all purchased from QIAGEN Inc. (Mississauga, Ontario). Ultrapure agarose was purchased from Invitrogen. Agarose gel electrophoresis (1.0 in Tris-borate-EDTA (TBE) containing 5 μL EtBr/100 mL) was conducted with TBE buffer (Sigma) at 75 V. The fusion of LtnJ

and MBP protein in *E. coli* was done using the Protein Fusion & Purification (pMAL™) System #E8000S (New England Biolabs Inc.).

4.2.3. Purification of Recombinant Proteins

Pilot expression and purification of recombinant proteins were conducted according to protocols detailed in the pMAL™ Protein Fusion and Purification and the QIAexpressionist™ (QIAGEN Inc.) utilizing the media that was appropriate to each individual transformant. The pertinent steps were monitored by SDS-PAGE gel electrophoresis. Protease Inhibitor Cocktail Tablets (cφmplete™ EDTA-free and cφmplete™ mini EDTA-free) were purchased from Roche (Roche Diagnostics Canada, Laval Quebec). IPTG was supplied by Rose Scientific Ltd. Amylose resin and Factor Xa was obtained from New England Biolabs Inc. Ni-NTA Superflow and Xa Removal Resin were purchased from QIAGEN Inc. (Mississauga, Ontario). BSA used for Bradford assays was purchased from Sigma Diagnostics. Unless specified otherwise, all centrifuge steps were done on either a Sorvell® RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Newton, CT, USA) or a Centrifuge 5415D (Ependorf, Hamburg, Germany).

4.2.4. SDS-PAGE, Western Blot, and Dot Blot Analysis

The proteins were resolved on either 10% or 12% (w/v) acrylamide gels.[180] Electrophoresis was done at a constant voltage (150 V) until tracking dye front (Laemmli

Sample Buffer, Bio-Rad Laboratories) moved to the bottom of the gel. The gels were stained with Bio-safe Coomassie staining solution (Bio-Rad Laboratories). The molecular weight of each protein band was calculated with reference to a standard curve derived from the migration pattern of standard molecular weight markers.[181] All SDS-PAGE buffers, reagents, and gels were purchased from Bio-Rad Laboratories. DTT was supplied by Sigma.

For Western Blots, the polyacrylamide gel resolved proteins were electrophoretically transfered (30 V overnight) to a nitrocellulose membrane (pore size 0.45 μm, Bio-Rad Laboratories). Ponceau S stain (0.5% Ponceau S (Sigma) in 1% acetic acid) was used for visualization of the transferred proteins. The unoccupied sites on the nitrocellulose membrane were blocked with blotto [TBS (20 mM Tris, 137 mM NaCl, adjusted to pH 7.5 with 6 M HCl) containing 0.1% Tween 20 (Sigma-Aldrich) and 5% (w/v) non-fat-milk powder (Carnation)]. The nitrocellulose membrane was then incubated with either Anti-His_6-Peroxidase antibody (Roche Diagnostics Canada, Laval Quebec) (1:500 in TBS-Tween 20) for hexahistidine-tagged proteins or Anti-MBP (Rabbit) (New England Biolabs Inc.) (1:10 000 in TBS-Tween 20) for MBP-tagged proteins at 25 °C for 1 hour with gentle rocking followed by washing three times with TBS-Tween 20. For MBP-tagged proteins, the membrane was then incubated at 25 °C for 1 hour in Goat-Anti Rabbit HRP Conjugate (New England Biolabs Inc.) (1:4 000 in TBT-Tween 20) followed by washing three times with TBS-Tween 20. For both hexahistidine and MBP-tagged proteins, the membrane was then incubated in 3 mL freshly prepared substrate solution [SuperSignal® West Femto Maximum Sensitivity

Substrate (Pierce Biotechnology) or SuperSignal® HRP Substrate (Novagen)] for 0.5-5 minutes and developed onto CL-XPosure™ Film (Pierce Biotechnology).

For Western Dot Blot analysis, 1 μL of the fraction to be analyzed was spotted onto a nitrocellulose membrane (pore size 0.45 μm, Bio-Rad Laboratories). After allowing the membrane to dry, it was treated in a manner similar to that described above for Western Blots.

4.2.5. Transformation of *E. coli*

Both the desired competent *E. coli* cells and plasmids, stored at -70 °C, were thawed on ice (~5 min). 100 μL of the thawed competent cells was added to 50 ng of the desired plasmid and gently mixed to ensure dispersion and incubated on ice for 15 min. After a heat-shock treatment at 42 °C for 2 min, the mixture was incubated on ice for 2 min. LB broth (1 mL) was added and the resulting mixture was incubated at 37 °C, 240 rpm for 1 h. The *E. coli* transformants were then grown on LB agar, containing a selective concentration of appropriate antibiotic, at 37 °C for 20 h.

4.2.6. Plasmid Isolation and Sequencing

Tubes containing 10 mL LB with 50 μg/mL ampicillin and 1% glucose were inoculated with *E. coli* BL21(DE3) cells containing either plasmid pPC1 or pPC2 and

incubated at 37 °C overnight with shaking at 200 rpm. The plasmids were isolated from *E. coli* using a QIAprep™ Spin Miniprep Kit and sequenced using Amersham's DYEnamic ET terminator cycle sequencing premix kit with the T7 promotor and the T7 terminator primers. The DNA was purified by ethanol precipitation in the presence of sodium acetate/EDTA buffer. 2 μL sodium acetate/EDTA buffer and 80 μL ice-cold 95% ethanol was added to 20 μL of the sequenced DNA. The mixture was vortexed and incubated on ice for 10 min. The DNA pellet was collected by centrifugation (14 000 rpm, 15 min, 4 °C), washed with 1 mL 70 % ethanol, and dried at 37 °C for 10 min. Analysis was performed by acrylamide gel electrophoresis on an ABI 373 sequencer (Applied Biosystems) at the Alberta Peptide Institute (Department of Biochemistry, University of Alberta).

4.2.7. Construction of Plasmid pLS1A

The LtnJ containing fragment of the pPC1 plasmid was PCR amplified with the Platinum *Taq* High Fidelity DNA polymerase by using the primers OLS04 (5'-ATAATTCATATGAAGGCGATGGTTGCCATAAATC-3') and OLS03 (5'-TAATTGGATCCTTATGTATCATAAGAAGTATCATATC) (Sigma Genosys), containing the restriction sites *Nde*I and *Bam*HI, respectively (underlined). The PCR reaction contained 0.2 mM of each dNTP, 1.5 mM $MgSO_4$, 0.2 μM of each primer, 20 ng pPC1 template DNA, and 1.0 unit Platinum *Taq* Hi Fidelity DNA Polymerase in 1x High Fidelity PCR buffer (final volume 50 μL). The PCR cycle was as follows; 94 °C for 1 min, 3 cycles of 94 °C for 30 s, 48 °C for 30 s, 68 °C for 1 min and 10 s, then 27 cycles

of 94 °C for 30 s, 51.8 °C for 30 s, 68 °C for 1 min and 10 s, followed by 68 °C for 5 min. Formation of the PCR product was confirmed by agarose gel electrophoresis followed by purification using QIAquick™ PCR Purification Kit.

The PCR product was then cloned into the *Nde*I and *Bam*HI site of the pET-11a vector. Digestion of the PCR product was accomplished as follows; 5 units of *Nde*I, 5 units of *Bam*HI, and 630 ng PCR product were added to 1x React 3 buffer (final volume 20 μL) and the reaction was incubated at 37 °C for 2 hours. An additional 2.5 units *Nde*I was added and the reaction was incubated at 37 °C for another hour. 1040 ng pET-11a vector was digested using 7.5 units *Nde*I in 1x React 3 buffer (final volume 20 μL) and the reaction was incubated at 37 °C for 2 hours. 5 units *Bam*HI was added and the reaction was incubated at 37 °C for an additional 2 hours. The digested PCR product and pET-11a vector were isolated via agarose gel electrophoresis (1% Ultrapure agarose in TBE buffer). The gel was then stained with ethidium bromide, visualized on a UV light box and the DNA excised with a razor blade. The excised DNA fragments were purified from the agarose according to the QIAquick™ Gel Extraction Kit protocol using 30 μL elution buffer. Ligation of the PCR product (35 ng) into the pET-11a vector (64 ng) was done at room temperature with T4 DNA ligase (1 unit) in 1x T4DNA ligase buffer (final volume 20 μL). 2.5 μL of the ligation reaction was used to transform *E. coli* DH5α cells (50 μL). Plasmid DNA was isolated from transformants and digested with *Nde*I and *Bam*HI to confirm *ltnJ* insertion into pET-11a. The products were analyzed by agarose gel electrophoresis.

pLS1A was sequenced using Amersham's DYEnamic ET terminator cycle sequencing premix kit using the T7 promotor primer, the T7 terminator primer, the primer OLS09 (5′-CGTCCAGGAATTCCGACAAACTTTG-3′), and the primer OLS10 (5′-ACGGTACTTATATAACTTGTGGG-3′) (Biochemistry DNA Core Lab, University of Alberta). For each sequencing reaction, the DNA was purified by ethanol precipitation in the presence of sodium acetate/EDTA buffer. 2 μL sodium acetate/EDTA buffer and 80 μL ice-cold 95% ethanol was added to 20 μL of the sequenced DNA. The mixture was vortexed and incubated on ice for 10 min. The DNA pellet was collected by centrifugation (14 000 rpm, 15 min, 4 °C), washed with 1 mL 70 % ethanol, and dried at 37 °C for 10 min. Analysis was performed by acrylamide gel electrophoresis on an ABI 373 sequencer (Applied Biosystems) at the Alberta Peptide Institute (Department of Biochemistry, University of Alberta).

4.2.8. Construction of Plasmid pLS1B

The LtnJ containing fragment of the pPC1 plasmid was PCR amplified with the Platinum *Taq* Hi Fidelity DNA polymerase by using the primers OLS04 (5′-ATAATTCATATGAAGGCGATGGTTGCCATAAATC-3′) and OLS06 (5′-TAATTGGATCCTTAATGATGATGATGATGATGGGAACCACCTGTATCATAAG AAGTATCATATC-3′) (Sigma Genosys) containing the restriction sites *Nde*I and *Bam*HI, respectively (underlined). The PCR reaction contained 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.2 μM of each primer, 20 ng pPC1 template DNA, and 1.0 unit Platinum *Taq* Hi Fidelity DNA Polymerase in 1x High Fidelity PCR buffer (final volume 50 μL).

The PCR cycle, confirmation of the PCR product, and purification was the same as previously described in section 4.2.7.

The PCR product was then cloned into the *Nde*I and *Bam*HI site of the pET-11a vector. Digestion of the PCR product was accomplished as follows; 5 units of *Nde*I, 5 units of *Bam*HI, and 645 ng PCR product were added to 1x React 3 buffer (final volume 20 μL) and the reaction was incubated at 37 °C for 2 hours. An additional 2.5 units *Nde*I was added and the reaction was incubated at 37 °C for another hour. Digestion and isolation of the pET-11a vector was accomplished via agarose gel electrophoresis as described in section 4.2.7. Ligation of the PCR product (35 ng) into the pET-11a vector (64 ng) was done at room temperature with T4 DNA ligase (1 unit) in 1x T4DNA ligase buffer (final volume 20 μL). 2.5 μL of the ligation reaction was used to transform *E. coli* DH5α cells (50 μL). Plasmid DNA was isolated from transformants and digested with *Nde*I and *Bam*HI to confirm *ltn*J insertion into pET-11a. The products were analyzed by agarose gel electrophoresis.

pLS1B was sequenced using Amersham's DYEnamic ET terminator cycle sequencing premix kit using the T7 promotor, the T7 terminator, the primer OLS09 (5′-CGTCCAGGAATTCCGACAAACTTTG-3′), and the primer OLS10 (5′-ACGGTACTTATATAACTTGTGGG-3′) (Biochemistry DNA Core Lab, University of Alberta). For each sequencing reaction, the DNA was purified and analyzed as described in section 4.2.7.

4.2.9. Construction of Plasmid pLS2

Plasmid pLS2 was constructed in a manner similar to pLS1A using the primers OLS04 and OLS03 to amplify LtnJ and the vector pET-15b to clone the PCR fragment.

4.2.10. Construction of Plasmid pLS3

The LtnJ containing fragment of the pPC1 plasmid was PCR amplified with the Platinum *Taq* Hi Fidelity DNA polymerase by using the primers OLS05 (5′-ATGAAGGCGATGGTTGCCATAAATCC-3′) and OLS03 (5′-TAATTGGATCCTTATGTATCATAAGAAGTATCATATC-3′) (Sigma Genosys) containing the restriction site *Bam*HI (underlined). The PCR reaction contained 0.2 mM of each dNTP, 2 mM $MgSO_4$, 0.2 μM of each primer, 20 ng pPC1 template DNA, and 1.0 unit Platinum *Taq* Hi Fidelity DNA Polymerase in 1x High Fidelity PCR buffer (final volume 50 μL). The PCR cycle, confirmation of the PCR product, and purification was the same as previously described in section 4.2.7. Polishing the ends of the PCR product was accomplished using T4 DNA polymerase; 1.5 μg PCR product, 0.1 mM each dNTP, and T4 DNA polymerase (5 units) in 1x T4 DNA polymerase buffer (final volume 100 μL) and incubate at 11 °C for 15 min. The polished DNA was then purified by phenol extraction using 100 μL 1:1 phenol pH 6.6: chloroform/isoamyl alcohol (24/1) then 100 μL chloroform:isoamyl alcohol (24:1) followed by precipitation with 1/10 volume sodium acetate (3 M) pH 5.2 and 2.5 volumes ice-cold ethanol. The DNA pellet (collected via centrifugation, 14 000 rpm, 10 min) was washed with 1 mL 70% ethanol

(vortexed and collected via centrifugation, 14 000 rpm, 5 min), dried at 37 °C for 10 min, and resuspended in 17.5 μL H_2O.

The PCR product was then cloned into the *Xmn*I and *Bam*HI site of the pMAL-c2X vector. 1500 ng of the PCR product digested using 10 units of *Bam*HI in 1x React 3 buffer (final volume 20 μL) and the reaction was incubated at 37 °C for 2 hours. 1000 ng of pMAL-c2X was digested using 20 units *Xmn*I and 1x BSA in 1x NEBuffer 2 buffer (final volume 20 μL) and the reaction was incubated at 37 °C for 2 hours. After adding 1.5 μL of 1 M NaCl (to give 100 mM), 10 units *Bam*HI, and 15 μL NEBuffer 2 buffer, the reaction was incubated at 37 °C for an additional 2 hours. The digested PCR product and pMAL-c2X vector were isolated via agarose gel electrophoresis as described in section 4.2.7. Ligation of the PCR product (60 ng) into the pMAL-c2X vector (140 ng) was done at 14 °C overnight with T4 DNA ligase (1 unit) in 1x T4DNA ligase buffer (final volume 13 μL). 2.5 μL of the ligation reaction was used to transform *E. coli* DH5α cells (50 μL). Plasmid DNA was isolated from transformants and digested with *Eco*I and *Bam*HI to confirm *ltn*J insertion into pMAL-c2X. The products were analyzed by agarose gel electrophoresis.

pLS3 was sequenced using Amersham's DYEnamic ET terminator cycle sequencing premix kit using the primers MalE (5′-GGTCGTCAGACTGTCGATGAAGCC-3′), OLS09 (5′-CGTCCAGGAATTCCGACAAACTTTG-3′), and OLS10 (5′-

ACGGTACTTATATAACTTGTGGG-3′) (Biochemistry DNA Core Lab, University of Alberta). For each sequencing reaction, the DNA was purified and analyzed as described in section 4.2.7.

4.2.11. Detection of Soluble Production of LtnJ from *E. coli* BL21(DE3) and Origami™ B(DE3) Containing pPC1 or pPC2 at Different Temperatures

10 mL of LB broth containing 50 μg/mL ampicillin (or 15 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 50 μg/mL ampicillin for Origami™B(DE3) cells) and 1% glucose were inoculated with individual colonies from the *E. coli* strain of interest and incubated at 37 °C for 16 h at 200 rpm. The cells from 800 μL of this culture were collected via centrifugation (13 000 rpm, 1 min) and resuspended in 800 μL fresh TB broth containing 50 μg/mL ampicillin (or 15 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 50 μg/mL ampicillin) and 1% glucose. TB broth (50 mL in 250 mL Erlenmeyer flask) containing 50 μg/mL ampicillin (or 15 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 50 μg/mL ampicillin) and 1% glucose was inoculated with the re-suspended cells and incubated at 37 °C at 200 rpm until the culture reached an optical density of 0.6-0.8 at 600 nm. Production of the recombinant LtnJ was induced with IPTG, 1 mM. After induction the cultures were split into 5 X 10 mL and one of each of the cultures was incubated at 15 °C, 20 °C, 25 °C, 30 °C, or 37 °C at 200 rpm for 16 hours. The cells from 1 mL of each culture were collected by centrifugation (13 000 rpm, 6 min, 4 °C) and frozen at -20 °C overnight. To collect total and soluble protein fractions, cells were resuspended in 1 mL PBS (10.64 mM Na_2HPO_4, 1.34 mM KCl, 0.735 mM KH_2PO_4,

136.9 mM NaCl, adjusted to pH 7.4 with 1 M HCl) and 100 µL Popculture™ Reagent (Novagen) and 2 µL Lysonase™ Bioprocessing Reagent (Novagen) were added. After incubation at room temperature for 10 min, 20 µL and 5 µL were removed from each reaction for SDS-PAGE and Western Dot Blot analysis, respectively. These are the total protein fractions. To collect samples of the soluble protein fractions, the lysed reactions were centrifuged (13 000 rpm, 10 min, 4 °C) and 20 µL and 5 µL of the supernatant were collected. Each fraction was analyzed via SDS-PAGE and Western Dot Blots as described in section 4.2.4.

4.2.12. Detection of Soluble Production of LtnJ from *E. coli* BL21(DE3) Containing pLS1A, pLS1B, or pLS2 and *E. coli* K12 TB1, K12 JM101, K12 CAG629, K12 UT5600, K12 ER2507, and K12 ER2508 Containing pLS3 at Different Temperatures

4 mL of LB broth containing 50 µg/mL ampicillin and 1% glucose were inoculated with individual colonies from the *E. coli* strain of interest and incubated at 37 °C at 235 rpm until the culture reached an optical density of 0.5 at 600 nm. The cells from 3 mL of this culture were collected via centrifugation (13 000 rpm, 1 min) and re-suspended in 500 µL fresh TB broth containing 50 µg/mL ampicillin and 1% glucose. TB broth (70 mL in 250 mL Erlenmeyer flask) containing 50 µg/mL ampicillin and 1% glucose was inoculated with the resuspended cells and incubated at 37 °C at 235 rpm until the culture reached an optical density of 0.6-0.8 at 600 nm. Before induction, 1 mL of cells were collected by centrifugation (13 000 rpm, 6 min, 4 °C) and stored at -20 °C for Western Dot Blot and SDS-PAGE analysis (control sample).

Production of the recombinant LtnJ was induced with IPTG, 1 mM. After induction the cultures were split into 5 X 10 mL and one of each of the flasks was incubated at 15 °C, 20 °C, 25 °C, 30 °C, and 37 °C at 225 rpm for 16 hours for the 15 °C, 20 °C, 25 °C, and 30 °C cultures and 2 hours for the 37 °C culture. The cells from 1 mL of each culture were collected by centrifugation (13 000 rpm, 6 min, 4 °C) and frozen at -20 °C overnight. Cells from 1 mL of culture were also collected after 4 hours for the 30 °C culture. Total and soluble protein fractions were isolated as described in section 4.2.11.

4.2.13. Isolation of the N-terminal Histidine Tagged LtnJ from *E. coli* BL21(DE3) Containing pPC1

10 mL of LB broth containing 50 μg/mL ampicillin and 1% glucose was inoculated with *E. coli* BL21(DE3) containing pPC1 and incubated at 30 °C for 16 h at 200 rpm. The cells from the 10 mL culture were collected by centrifugation (10 000 rpm, 20 min), resuspended in 10 mL TB broth containing 50 μg/mL ampicillin and 1% glucose, and used to inoculate 500 mL of TB broth containing 50 μg/mL ampicillin and 1% glucose. The culture was incubated at 37 °C with shaking (200 rpm) until it reached an optical density of 0.6-0.8 at 600 nm, after which the production of the recombinant His_6-LtnJ was induced with IPTG, 1 mM. After induction the cultures were incubated at 20 °C, 200 rpm for 20 hours before the cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C).

0.5 g of the cells (from 1/10 of the growth culture) were resuspended in 3.5 mL ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0 adjusted with 1 M NaOH) containing one half of a cφmplete™ mini EDTA-free Protease Inhibitor Cocktail tablet. The cells were lysed using a French press and the lysate collected via centrifugation (pre-cooled 50 mL centrifuge bottles; pre-cooled centrifuge; 10 000 rpm, 20 min, 4 °C). The lysate was loaded onto a pre-washed (10 volumes of lysis buffer) 1 mL Ni-NTA resin column at a rate of 1 mL/min. The column was subsequently washed with 16 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0 adjusted with 1 M NaOH) at 1 mL/min. Elution of the His_6-tagged protein was accomplished using 3 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0 adjusted with 1 M NaOH). The presence of overexpressed His_6-LtnJ was monitored by SDS-PAGE gel electrophoresis (10%). Fractions containing the fusion protein were dialyzed (4 °C for 4 h, then transferred to fresh buffer and dialyzed for 10 h) against 50 mM NaH_2PO_4, 150 mM NaCl, pH 8.0 adjusted with 1 M NaOH using a 12 000-14 000 MWCO membrane (16 mm diameter, vol/length = 2 mL/cm, flat width 25±2 mm, Fisher Scientific). The Bradford Assay was used to determine the total protein concentration and the solution was concentrated via centrifugation (4 °C, 10 000 MWCO Amicon micron filter, 3000 rpm) until the protein concentration was 0.5 mg/mL (~1 h).

4.2.14. Isolation of the Maltose Binding Protein-LtnJ Fusion from *E. coli* K12 ER2507 Containing pLS3

6 mL LB broth containing 100 μg/mL ampicillin was inoculated with *E. coli* K12 ER2507 containing pLS3 and incubated at 30 °C for 16 h at 225 rpm. Fermentations were done on a one litre scale with 500 mL of growth media per 2 L Erlenmeyer flask. The sterile LB broth contained; 100 μg/mL ampicillin, 0.2% glucose, 0.1 mM $ZnSO_4 \cdot 7H_2O$, 0.3 mM $FeSO_4 \cdot 7H_2O$, 0.01 mM $CoCl_2 \cdot 6H_2O$, 0.01 mM $NiSO_4 \cdot 6H_2O$, 0.01 mM $CuSO_4 \cdot 5H_2O$, 1 mM $MgSO_4$, 0.2 mM $MnSO_4 \cdot 4H_2O$, 0.01 mM $Na_2MoO_4 \cdot 2H_2O$, and 0.1 mM $CaCl_2$. Each 500 mL of LB broth was inoculated with 6 mL *E. coli* K12 ER2507 culture and incubated at 37 °C with shaking (225 rpm) until the cultures reached an optical density of 0.5 at 600 nm. Production of the recombinant MBP-LtnJ was induced with IPTG, 0.3 mM. After induction the cultures were incubated at 30 °C, 225 rpm for 4 hours before the cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C).

The cells were pooled and resuspended in 50 mL ice-cold column buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.4 adjusted with 1 M HCl) containing one cφmplete™ EDTA-free Protease Inhibitor Cocktail tablet. The resuspended cells were frozen at -20 °C overnight, defrosted in ice water, and lysed using a French press. The lysate was collected via centrifugation (pre-cooled 250 mL centrifuge bottles; pre-cooled centrifuge; 8000 rpm, 20 min, 4 °C), diluted with column buffer to 300 mL, and loaded onto a pre-washed (10 volumes of column buffer) 15 mL amylose resin column at a rate of 1 mL/min. The column was subsequently washed with 700-800 mL of column buffer at 1

mL/min. Elution of the MBP-fusion protein employs the same buffer containing 10 mM maltose. 3 mL fractions were collected on a Bio-Rad fraction collector with UV monitoring capability. The presence of overexpressed MBP-LtnJ was monitored by SDS-PAGE gel electrophoresis (10%). Fractions containing the fusion protein were dialyzed (4 °C for 4 h, then transferred to fresh buffer and dialyzed for 10 h) against Factor Xa cleavage buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2, pH 7.5 adjusted with 1 M HCl) using a 12 000-14 000 MWCO membrane (16 mm diameter, vol/length = 2 mL/cm, flat width 25±2 mm, Fisher Scientific). The Bradford Assay was used to determine the total protein concentration and the solution was concentrated via centrifugation (4 °C, Amicon Ultra 50 000 MWCO (Millipore), 3000 rpm) until the protein concentration was 1 mg/mL (~15 min).

4.2.15. Cleavage of LtnJ from the Maltose Binding Protein Fusion

The cleavage reaction was done at a w/w ratio of 2% Factor Xa with respect to the mass of the fusion protein (e.g., 1 mg Factor Xa/50 mg fusion protein) with gentle mixing at 4 °C for 6 h. The Factor Xa was removed using Xa removal resin and any remaining Factor Xa was irreversibly inhibited by adding AEBSF (1.6 μM). The solution was then dialyzed (4 °C for 4 h, then transferred to fresh buffer and dialyzed for 10 h) against either 50 mM Tris-HCl, 200 mM NaCl, pH 5.5 (adjusted with 1 M HCl) or 50 mM NaH_2PO_4, 200 mM NaCl, pH 5.5 (adjusted with 1 M HCl) using a 12 000-14 000 MWCO membrane (16 mm diameter, vol/length = 2 mL/cm, flat width 25±2 mm, Fisher Scientific). The Bradford Assay used to determine total protein concentration.

4.2.16. Preparation of Cell Free Extract from *L. lactis* subsp. *lactis* MG1363

Lactococcus lactis subsp. *lactis* MG1363 cells were grown and harvested as described in section 4.3.5. 10 g of cells were resuspended in 100 mL of 50 mM Tris-HCl, 100 mM NaCl, pH 5.5 (adjusted with 1 M HCl) containing two cοmplete™ EDTA-free Protease Inhibitor Cocktail tablets. Cells were lysed using a French press and the lysate was collected via centrifugation (4 °C, 8000 rpm, 20 min).

4.2.17. Isolation of Lacticin 3147 Mutants from an *L. lactis* Δ*ltnJ* Mutant

Lacticin 3147 mutants from *Lactococcus lactis* subsp. *lactis* DPC3147 Δ*ltnJ* were isolated using a modified procedure from that previously published in N. Martin's PhD Thesis.[3]

10 mL of M17 (Difco) broth was inoculated with *Lactococcus lactis* subsp. *lactis* DPC3147 Δ*ltnJ* and incubated for 24 hours at 30 °C. The culture was used to inoculate 4 L (divided into four 1 L flasks) containing a modified tryptone-yeast broth[182] and incubated at 30 °C for 16 to 24 hours. The modified tryptone-yeast media contained, per 800 mL; tryptone 2.5 g, yeast extract 5.0 g, 1.5 g D/L- methionine, 50 mg $MnSO_4 \cdot 4H_2O$, and 125 mg $MgSO_4$. Prior to sterilization the media was passed through a column (2.5 x 40 cm) packed with 75 g of Amberlite XAD-16 resin (Aldrich) to remove hydrophobic components that would otherwise interfere with the isolation of the hydrophobic peptide. The Amberlite XAD-16 resin was pre-treated by washing with one column volume of

70% isopropanol (pH 2 by addition of 1 M HCl) followed by 5 column volumes of H_2O. After dividing the 3.2 litres of clarified media into separate 800 mL volumes it was sterilized (15 min. at 121° C), as were separate solutions of D-glucose (100 g/L) and β-glycerophosphate (190 g/L). The D-glucose and β-glycerophosphate solutions were sterilized separately to prevent caramelization of the media, making the concomitant peptide isolation less problematic. After sterilization of the media, 100 mL of both the glucose and β-glycerophosphate solutions was added to each 800 mL media solution to achieve a final volume of 1 L.

After a growth time of 16 to 24 hours, 50 g of ammonium sulfate and 75 mg DTT was added to each one litre fermentation. The cells and precipitated proteins were collected via centrifugation (8000 rpm, 20 min, 4 °C). The mutant peptides were extracted with 250 mL 70% IPA (adjusted to pH 2 with 1 M HCl), stirring at 4 °C for 3 hours. The cell debris was removed via centrifugation (8000 rpm, 20 min, 4 °C) and the supernatant reduced to a volume of 20 mL by rotary evaporation and lyophilized. The sample was prepared for MALDI-TOF analysis using a ZipTip® pipette tip (Millipore), eluting the mutant lacticin 3147 peptides with 2 μL 50% isopropanol. A mutant solution was prepared by resuspending the crude lacticin 3147 mutants in 2 mL of 10% isopropanol.

4.2.18. His_6-LtnJ Activity Assay with Dipeptidyl Substrates using *E. coli* BL21(DE3) containing pPC1

Each of the synthesized dipeptides **24**, **25**, and **26** were tested as substrates of His_6-LtnJ. The assay conditions were as follows; 100 μg His_6-LtnJ, 100 nmol NADH or 100 nmol NADPH, and 50 nmol dipeptidyl substate **24**, **25**, or **26** in either a total volume of 200 μL Tris or 200 μL NaH_2PO_4 buffer. A control assay was prepared for each dipeptide as follows; 100 μg His_6-LtnJ, 100 nmol NADH, 100 nmol NADPH, 50 nmol dipeptidyl substrate **24**, **25**, or **26**, and 50 nmol dipeptidyl D-alanine containing product **34**, **35**, or **36** in either a total volume of 200 μL Tris or 200 μL NaH_2PO_4 buffer. The reactions were incubated at 30 °C for 6 hours, heated to 85 °C for 5 minutes to kill any remaining active enzyme, and lyophilized. The dipeptides were extracted with 500 μL methanol and centrifuged (2 X 13 000 rpm, 5 minutes) to remove insoluble material. Each extraction was analyzed via electrospray mass spectrometry (40, 80, and 120 eV) in order to detect the formation of the D-alanine containing dipeptides **34**, **35**, and **36**.

4.2.19. LtnJ Activity Assay with Dipeptidyl Substrates using *E. coli* K12 ER2507 containing pLS3 or CFE of *L. lactis*

Each of the synthesized dipeptides **24**, **25**, and **26** were tested as substrates of LtnJ purified from the MBP fusion. The assay conditions were as follows; 100 μg LtnJ in either Tris or NaH_2PO_4 buffer (total volume 200 μL) or 4 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 100 nmol NADH, 100 nmol NADPH, 50 nmol dipeptidyl substate

24, **25**, or **26** and either none or one of the following co-factor combinations (final concentrations): 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 0.3 μM $FeSO_4 \cdot 7H_2O$ and 1 mM FAD, 1 mM EDTA, 1 mM DTT. A control assay was prepared for each dipeptide as follows; 100 μg LtnJ in either Tris or NaH_2PO_4 buffer (total volume 200 μL) or 4 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 100 nmol NADH, 100 nmol NADPH, 50 nmol dipeptidyl substrate **24**, **25**, or **26**, 50 nmol dipeptidyl D-alanine containing product **34**, **35**, or **36**, and final concentrations of 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1.0 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, 1 mM EDTA, and 1 mM DTT. The reactions were incubated at 30 °C for 6 hours, heated to 85 °C for 5 minutes to inactivate any remaining active enzyme, and lyophilized. The dipeptides were extracted with 500 μL methanol and centrifuged (2 X 13 000 rpm, 5 minutes) to remove insoluble material. Each extraction was analyzed via electrospray mass spectrometry (40, 80, and 120 eV) in order to detect the formation of the D-alanine containing dipeptides **34**, **35** and **36**.

4.2.20. LtnJ Activity Assay with Lacticin 3147 Δ*ltnJ* Mutants using *E. coli* K12 ER2507 containing pLS3

The lacticin 3147 Δ*ltnJ* mutants were tested as substrates of LtnJ purified from the MBP fusion. The assay conditions were as follows; 100 μg LtnJ in either Tris or NaH_2PO_4 buffer (total volume 200 μL), 100 nmol NADH, 100 nmol NADPH, 60 μL lacticin 3147 Δ*ltnJ* mutant solution (preparation detailed in section 4.2.17.), and either none or one of the following co-factor combinations (final concentrations): 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 0.3 μM $FeSO_4 \cdot 7H_2O$ and 1 mM FAD,

1 mM EDTA, 1 mM DTT. A control assay was prepared as follows; 100 nmol NADH, 100 nmol NADPH, and 60 μL lacticin 3147 Δ*ltnJ* mutant solution (preparation detailed in section 4.2.17.) in a total volume of 200 μL Tris buffer. The reactions were incubated at 30 °C for 6 hours, heated to 85 °C for 5 minutes to inactivate any remaining active enzyme, and lyophilized. The lacticin 3147 mutant peptides were extracted with 50 μL IPA and centrifuged (2 X 13 000 rpm, 5 minutes) to remove insoluble material. The solvent was removed and each sample was reconstituted in 3 μL of water (0.1% TFA). Each extraction was analyzed via MALDI-TOF mass spectrometry using a ZipTip® pipette tip in order to detect the formation of lacticin 3147.

4.2.21. LtnJ Activity Assay with Tripeptidyl Substrates using LtnJ from *E. coli* K12 ER2507 containing pLS3 or CFE of *L. lactis*

Each of the synthesized tripeptides **43**, **53**, and **54** were tested as substrates of LtnJ. The assay conditions were as follows; 100 μg LtnJ in either Tris or NaH_2PO_4 buffer (total volume 200 μL) or 9 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 100 nmol NADH and 100 nmol NAD^+ or 100 nmol NADPH and 100 nmol $NADP^+$, 50 nmol tripeptidyl substate **43**, **53**, or **54** and either none or all of the following co-factors (final concentrations): 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, and 1 mM FMN. A control assay was prepared for each tripeptide as follows; 100 μg LtnJ in either Tris or NaH_2PO_4 buffer (total volume 200 μL) or 9 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 100 nmol NADH, 100 nmol NAD^+, 100 nmol NADPH, 100 nmol $NADP^+$, 50 nmol tripeptidyl substate **43**, **53**, or **54**, 50 nmol tripeptidyl D-alanine

containing product **58**, **59**, or **60**, and final concentrations of 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, and 1 mM FMN. The reactions were incubated at 30 °C for 6 hours and lyophilized. The dipeptides were extracted with 500 μL methanol and centrifuged (2 X 13 000 rpm, 5 minutes) to remove insoluble material. Each extraction was analyzed via LCMS RP-HPLC using a steel walled C_{18} column (Luna (Phenomenex), 50 x 2 mm, 3 μm) in order to detect the formation of the D-alanine containing tripeptides **58**, **59**, and **60**. In the LCMS method employed a 10 μL injection was applied and a gradient of water and methanol was used to separate and analyze the presence of each tripeptide. Gradient; 15% methanol to 95% in 10 min, holding at 95% for 10 min, returning to 15% in 0.1 min and remaining at this concentration for an additional 10 min (flow rate 0.25 mL/min, detection at 120 eV). Compound **54** R_f=11.1 min, compound **60** R_f=10.9 min, compound **43** R_f=9.2 min, compound **58** R_f=8.8 min, compound **53** R_f=11.4 min, compound **59** R_f=11.2 min.

4.2.22. Detection Limits of LCMS

The following mixtures were prepared and analyzed via LCMS (method described in section 4.2.22) for each tripeptidyl substrate **43**, **53**, and **54**; mixture 1 contained 9 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, 450 nmol tripeptidyl substate **43**, **53**, or **54**, 50 nmol tripeptidyl D-alanine containing product **58**, **59**, or **60**, and final concentrations of 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, and 1 mM FMN, mixture

2 contained 9 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, 450 nmol tripeptidyl substate **43**, **53**, or **54**, 5 nmol tripeptidyl D-alanine containing product **58**, **59**, or **60**, and final concentrations of 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, and 1 mM FMN, and mixture 3 contained 9 mL of CFE from *L. lactis* subsp. *lactis* MG1363, 2 μmol NADH, 2 μmol NAD^+, 2 μmol NADPH, 2 μmol $NADP^+$, 450 nmol tripeptidyl substate **43**, **53**, or **54**, 0.5 nmol tripeptidyl D-alanine containing product **58**, **59**, or **60**, and final concentrations of 0.1 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $MgSO_4$, 0.3 μM $FeSO_4 \cdot 7H_2O$, 1 mM FAD, and 1 mM FMN. Results displayed in section 2.14.

4.3. Bacteriocins as Spermicides

4.3.1. Bacterial Strains, Plasmids, and Culture Media

The plasmids and producer strains utilized in the study of bacteriocins as spermicides are listed in Table 6. The overproducing strain, *Lactococcus lactis* subsp. *lactis* MG1363 containing pMRC01 and pOM02 was obtained from Hill and Ross and contains the original lacticin producing plasmid, pMRC01 and a second plasmid, pOM02 that is a pCI372 carrying the lacticin genes.[118] The overproducing strain was grown at 30°C without aeration in M17 broth supplemented with 0.5% (wt/vol) lactose. *L. lactis* subsp. *cremoris* HP was used as a standard sensitive strain and was cultured in Difco M17 broth (supplemented with 0.5% (wt/vol) lactose) at 30°C without aeration. Bacterial strains, stored in M17 broth supplemented with 20% (v/v) glycerol, were maintained as frozen stock cultures at -80 °C.

Table 6. Bacterial strains and plasmids used in bacteriocins as spermicides project.

Strain or plasmid	Description	Reference or source
Strains		
L. lactis subsp. *lactis* DPC3147	Lacticin 3147 producer strain	[109]
L. lactis subsp. *lactis* MG1363	Host strain for lacticin 3147 overexpression	[160]
L. lactis subsp. *cremoris* HP	Lacticin 3147 and nisin indicator organism	[173]
Plasmids		
pMRC01	60.2-kb natural lactococcal plasmid containing *ltnEFIRA1A2M1TM2J*	[119]
pOM02	pCI372-*ltnEFIRA1A2M1TM2J*	[173]

4.3.2. Antimicrobial Activity Monitoring During Purification

Antimicrobial activity was monitored by inhibition of indicator strain growth on agar plates. Plates were prepared by inoculating 200 mL of molten (48°C) M17 Agar (40.0 g/L) with 1.0 mL of an overnight culture of indicator organism *L. lactis* subsp. *cremoris* HP. The molten agar was swirled gently and then dispensed in 20 mL aliquots onto sterile Petri plates, allowed to cool, and stored at 4°C. When performing activity assays, small wells (4.6 mm diameter) were made in the seeded agar plates, and 50 μL aliquots of the solutions to be tested were dispensed into each well. The plates were then incubated at 30°C for 24 hours.

4.3.3. Mass Spectrometry

Mass spectrometric analyses were performed with a single-stage reflectron, linear matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems Voyager Elite). All spectra were recorded in positive ion mode with an acceleration voltage of 20 kV in the presence of a nitrogen laser (λ = 337 nm) used for desorption/ionization of the samples. Samples were prepared using sinapinic acid (Aldrich) as a matrix. Solutions containing the sample peptide were mixed in even part with a stock solution of sinapinic acid (10 mg/mL) in 50% acetonitrile (0.1% TFA). A thin layer of sinapinic acid was deposited on the surface of a gold or stainless steel target by delivery of a small droplet (0.6 μL) of a solution containing sinapinic acid (5 mg/mL) in 3:2 acetone:methanol. After evaporation of the acetone/methanol, a 0.4 μL

droplet of the solution containing the sample peptide-matrix mixture was deposited on top of the fresh matrix layer on the plate. The solvent was evaporated at 1 atm prior to analysis.

4.3.4. Isolation of Nisin A (16)

A 2.5% preparation of nisin was purchased (Sigma) where the majority of contaminants are sodium chloride and other small peptides. Purification of nisin was achieved using reverse phase RP-HPLC. 250 mg of the commercial material was resuspended in 10 mL 35% acetonitrile (0.1% TFA) and sonicated for 15 minutes. The insoluble material was then removed by centrifugation (4 °C, ~8000 rpm (setting 7), 10 min using an IEC Clinical Centrifuge, Damon/IEC Division, Heedham, MA, USA) the supernatant was injected onto a steel walled RP-HPLC column (Grace Vydac, 22 x 250 mm, 10 μm). In the method employed, a 1.0 mL injection was applied and a gradient of water and acetonitrile (0.1% TFA) was used to separate and isolate the peptide. Gradient; 30% acetonitrile for 5 min, then climbing to 38.8% in 11 min returning to 30% in 0.5 min and remaining at this concentration for an additional 5 min (flow rate 15 mL/min., detection at 220 nm). Nisin was isolated as a single peak (R_t=13.0 min.) and its identity confirmed by MALDI-TOF mass spectrometry (Figure 58).

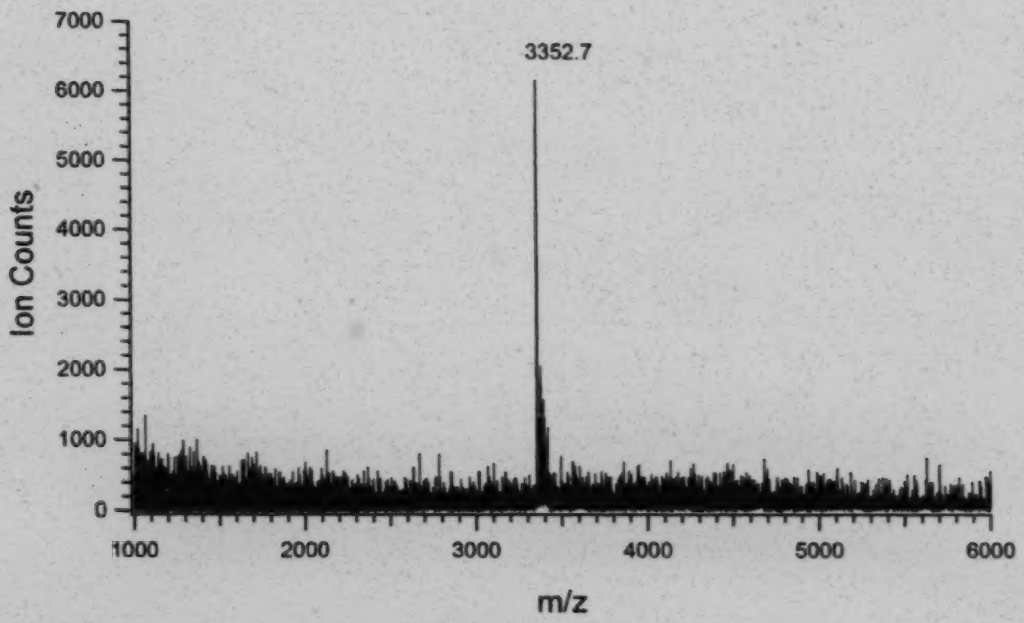


Figure 58. MALDI-TOF mass spectrum of nisin.

Approximately 3 mg of pure nisin was thus obtained with an efficiency of recovery near 50%.

4.3.5. Isolation of Lacticin 3147 (18, 19)

Lacticin 3147 was isolated from the cells of the overproducing strain *Lactococcus lactis* subsp. *lactis* MG1363 containing pMRC01 and pOM02. 10 mL culture tubes of M17 broth were inoculated with *Lactococcus lactis* subsp. *lactis* MG1363 and grown for 24 hours at 30°C. A total of 4 L of modified tryptone-yeast media was used for fermentation as described in section 4.2.17.

Each 1 L volume of fermentation media was inoculated with a 10 mL *Lactococcus lactis* subsp. *lactis* MG1363 preculture (1% inoculum). After a total growth time of 16 to 24 hours at 30°C without aeration, 50 g of ammonium sulfate and 75 mg of DTT was added to each 1 L fermentation. From this point on, all buffers used in the isolation procedure contained 1 mM DTT to protect the lantibiotic peptides from oxidation. The cells from the 4 L fermentation were collected by centrifugation (20 min., 8000 rpm), combined, and resuspended in 250 mL of 70% isopropanol (adjusted to pH 2 by addition of 1M HCl). After stirring for 3 hours at 4 °C the cell debris was removed by centrifugation (20 min., 8000 rpm) and the supernatant reduced to a volume of 60 mL by rotary evaporation. The concentrated bacteriocin solution was next loaded onto a disposable column containing 10 g of C_{18} silica (Varian C_{18} megabond elut). Prior to sample loading, the C_{18} silica was pre-equilibrated with 60 mL of 100% methanol. After loading the sample the column was washed successively with 60 mL of purified (milli-Q system, Millipore, Bedford, Mass.) water, 60 mL of 30% ethanol, and 40 mL of 25% isopropanol. The active peptides were removed from the column by washing with 100 mL of 70% isopropanol (pH 2 by the addition of 1M HCl). The 70% isopropanol fraction was then reduced to a 20 mL volume by rotary evaporation and lyophilized.

The lyophilized sample was then dissolved in 10 mL of 40% isopropanol and the lacticin 3147 A1 and A2 peptides were isolated by reverse phase RP-HPLC using a steel walled column (Grace Vydac, 22 x 250 mm, 10 μm). In the method employed a 1.0 mL injection was applied and a gradient of water and isopropanol (0.1% TFA) was used to separate and isolate each peptide. Helium was bubbled through the RP-HPLC solvents

throughout the purification in order to prevent oxidation of the peptides. Gradient; 24% isopropanol for 5 min, then climbing to 44% in 20 min returning to 24% in 0.5 min and remaining at this concentration for an additional 5 min (flow rate 12 mL/min., detection at 220 nm). The LtnA1 peptide was isolated as a single peak (R_t=17.0 min.) and the LtnA2 peptide was isolated as a single, broad peak (R_t=22.7 min.). The active fractions for each peptide were pooled and stored on ice in a glove bag filled with argon until separation was completed. Each pooled fraction was reduced to a total volume of 5 mL *in vacuo*, using an argon balloon to restore the pressure in the rotary evaporator to 1 atm. The concentrated samples were transferred to vials in a glove bag filled with argon, frozen, and lyophilized. The identity of each peptide was confirmed by MALDI-TOF mass spectrometry (Figures 59 and 60).

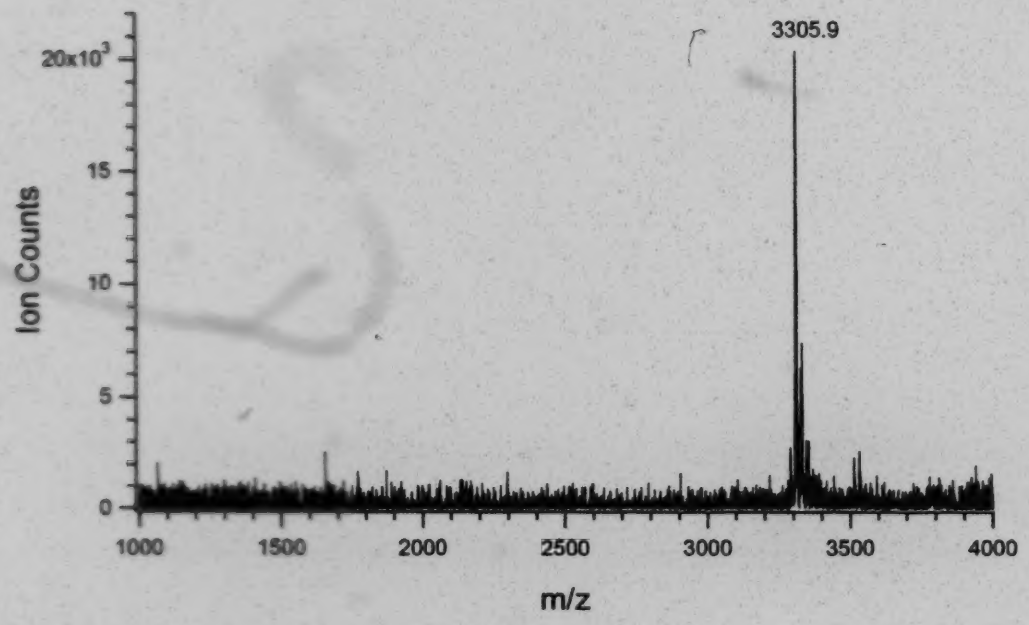


Figure 59. MALDI-TOF mass spectrum of LtnA1.

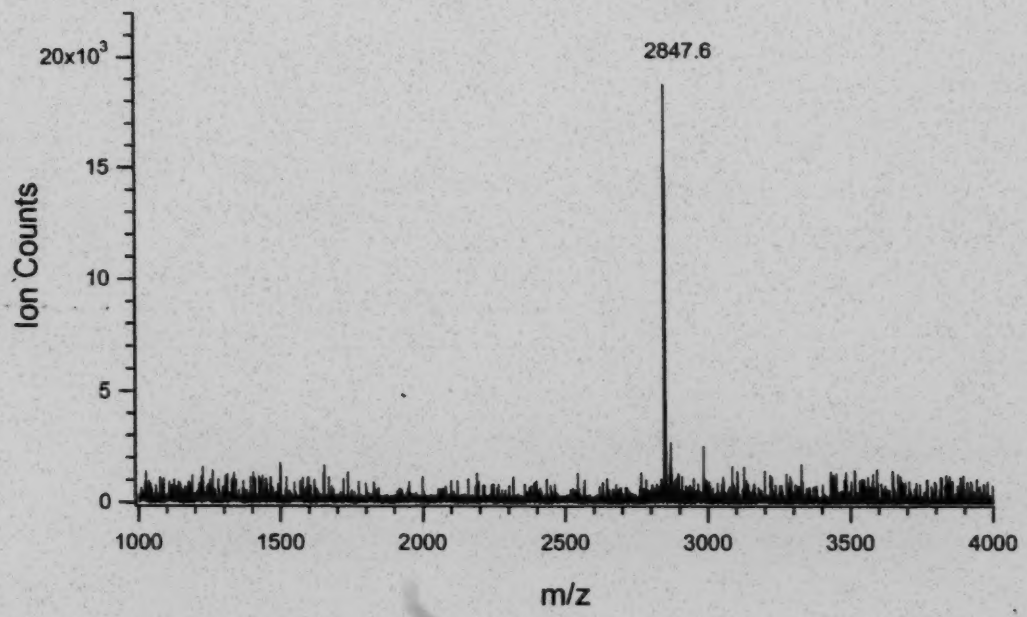


Figure 60. MALDI-TOF mass spectrum of LtnA2.

Approximately 2.0 to 4.0 mg of each peptide was recovered from 4 litres of culture. All purified samples were stored under argon at –80 °C to prevent the very rapid oxidation of the peptides.

4.3.6. Collection of Semen Samples

Semen samples were collected from Simmental bulls by penile electroejaculation (Leduc Veterinary Hospital, Leduc, AB), from Percheron stallions or Welsh Mountain ponies by artificial vagina (D&R Veterinary Services, Wetaskawin, AB), from Large White or Duroc boars by manually assisted ejaculation (University of Alberta Swine Research and Technology Centre, Edmonton, AB), and from Long-Evans rats by surgical

extraction as described in section 4.3.7. (Dr. Jacobs-Kaufman's Laboratory, University of Alberta Department of Physiology & Medicine, Edmonton, AB). The semen collected from Percheron stallions or Welsh Mountain ponies was filtered prior to use (performed by D&R Veterinary Services). All semen sample concentrations were adjusted with testing medium to yield sperm count ranging from 75-122 X 10^6/mL and had a minimum of 70% motility, 43% morphology, and 50% viability.

4.3.7. Collection and Analysis of Rat Sperm

A motile population of spermatozoa was surgically excised from the cauda epididymis of a sexually mature Long-Evans rat in a modified diffusion method as described by Klinefelter *et al.*[153] Prior to surgery, the rat was anesthetized using intra-peritoneal injection of pentobarbital (62 mg/kg/b wt.), intra-muscular injection of atropine (0.10 mg/kg/b wt.), and sub-cutaneous injection of inactin (80 mg/kg/b wt.) (administered 30-45 min. after sodium pentobarbital). The body temperature of the rat was maintained at 37 °C for the entire procedure using a deltaphase heat pad. In addition, the animal was covered with a flannel blanket and warmed with an overhead heat lamp. The animal's body temperature (monitored with a rectal probe) and mean arterial pressure were measured online (PowerLab software, ADInstruments, Australia). The rat received 3 mL/hr of a physiological saline infusion intravenously. The genital region was shaved with a razor blade (Personna Medical, Virginia, USA) and swabbed with povidone iodine disinfectant solution (7.5%, Purdue Pharma, Ontario, Canada). A midline incision was made through the scrotum and the left testicle was exposed by blunt

dissection of the surrounding tissues. The connective tissue binding the cauda epididymis to the testicle was removed with fine tissue scissors. The cauda epididymis was then gently moved aside to a platform covered in moist gauze, the area being kept warm by the deltaphase heat pad underneath and the heat lamp overhead. Excepting the cauda epididymis, exposed tissues in the groin area were covered with moist gauze, plastic wrap and a flannel blanket to maintain 37 °C body temperature. The outer connective tissue of the cauda epididymis was then removed gently with fine tissue scissors under a dissecting microscope (Zeiss), exposing epididymal tubules containing sperm. After rinsing in warm Hank's balanced salt solution (Gibco, catalog #14065-056 supplemented with 0.35 mg/mL sodium bicarbonate, 4.2 mg/mL HEPES (Sigma), 0.9 mg/mL D-glucose, 0.1 mg/mL sodium pyruvate, 0.025 mg/mL soybean trypsin inhibitor (Sigma), and 2.0 mg/mL BSA (Sigma), pH 7.4 as described by Slott *et al.*[154]), the epididymis was submerged in a small container (2.5 cm diameter, 0.5 cm depth) filled with the aforementioned medium. The vas deferens, accessed through a midline abdominal incision, was clamped with blunt hemostats to engorge the sperm filled tubules of the cauda epididymis. A series of small incisions were made in individual tubules (while submerged in medium) with fine tissue scissors. Live sperm cells were then allowed to stream and diffuse into the medium. The resulting concentrated sperm solution was immediately transferred to warm (37 °C) glass vials and stored in a 37 °C incubator until needed for subsequent analysis and bacteriocin testing. If required the contralateral cauda epididymis was isolated and the surgical procedure was repeated.

Sperm motility was determined by counting the numbers of motile and immotile spermatozoa in several randomly selected fields under a 400X microscope objective with the aid of a Laboratory Counter (Fisher). The number of progressively motile spermatozoa was counted first, counting only those spermatozoa that were in the field at one moment in time, followed by the number of non-progressively motile spermatozoa and immotile spermatozoa present within the same field. At least 200 spermatozoa were counted in total and the percentage of motile cells calculated.

The concentration of the collected sperm sample was determined using a haemocytometer. The sperm was diluted 20 times using 1 M HCl as a fixative. 10 μL of the diluted solution was spotted onto each grid on the haemocytometer and the cells were allowed to settle for 5 minutes. A Tally Counter (Fisher) was used to count the number of sperm heads in 5 of the 25 squares. If a spermatozoon was lying on the line dividing two adjacent squares then it was only counted if it was on the upper or left-hand side of the square being counted. The count was repeated on the second chamber and the mean of these two counts was used to calculate the concentration of sperm in the original sample. The process was repeated if the counts were not within 10% of each other. The sperm concentration was then calculated using the following formula; concentration = N X 5 X 20 X 10^4 sperm/mL where N is the mean number of spermatozoa counted in 5 squares.

The morphology of the sample was determined by preparing an eosin-nigrosin stain. With the aid of a Laboratory Counter (Fisher) the numbers of normal and abnormal

sperm were counted until a minimum of 200 spermatozoa had been counted. The percentage of normal sperm was determined.

The vitality of the sample was calculated as follows; % vitality = (concentration of sperm X %motility X %morphology) / (concentration of sperm).

4.3.8. In Vitro Testing of Peptides on Sperm Motility

Two-fold serial dilutions of nisin, LtnA1, LtnA2, and subtilosin A(800, 400, 200, 100, 50, 25 μg) were prepared in either Hank's balanced salt solution (Gibco, catalog #14065-056 supplemented with 0.35 mg/mL sodium bicarbonate, 4.2 mg/mL HEPES (Sigma), 0.9 mg/mL D-glucose, 0.1 mg/mL sodium pyruvate, 0.025 mg/mL soybean trypsin inhibitor (Sigma), and 2.0 mg/mL BSA (Sigma), pH 7.4) or Kreb's-Ringer buffer (3 mM KCl, 145 mM NaCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1,.6 mM Na_2HPO_4, 0.4 mM $NaH_2PO_4 \cdot H_2O$, pH 6.8 adjusted with 1 M HCl). Dose- and time-dependent effects of nisin, LtnA1 and LtnA2 (individually and together), and subtilosin A on sperm motility of rat, horse/pony, bull, and pig were determined in vitro by Sander-Cramer assay.[150] In this assay, 1 mL of a solution containing the bacteriocin in various concentrations is added to 200 μL of semen (both pre-heated to 37 °C), using a pipet and at the moment of contact, the stop-watch is started. The resulting solution is mixed rapidly 5 times with the pipet and a hanging-drop is prepared and viewed under 400x magnification with a microscope equipped with a heated platform (37 °C). When no motion, even vibratory, is seen in any sperm in five consecutive fields, the time is recorded. Time is recorded in ¼

minute intervals to 5 minutes and after that, is recorded to the nearest minute. The reading is confirmed by preparation of a second hanging-drop followed by observation of sperm motility. If sperm motility is seen in the second slide, the procedure is repeated until confirmation of the reading is obtained. 500 μL of all test samples containing sperm and bacteriocin were added to 500 μL of buffered glucose (30 mg/mL D-glucose, 2.4 mg/mL Na_2HPO_4, 2 mg/mL NaCl, 0.1 mg/mL KH_2PO_4) and incubated for 30 min at 37 °C. Sperm motility in 10 fields was observed under a microscope (400X objective) and samples showing no sign of movement were recored as "pass." In addition, sperm viability was done for each sample by eosin-nigrosin staining.[152] All experiments were done in duplicate.

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